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Studies on the Metabolism of the Protozoa

4. METABOLISM OF THE PARASITIC FLAGELLATE *STRIGOMONAS ONCOPELTI**

By J. F. RYLEY

Imperial Chemical (Pharmaceuticals) Limited, Biological Laboratories, Morley, Wilmslow, Cheshire

(*Received* 12 *August* 1954)

Strigomonas oncopelti is a flagellate of the family Trypanosomidae parasitic in the latex of the common milkweed, being transmitted from plant to plant by the bite of hemipterous insects. Several strains of the parasite were isolated in bacteria-free culture by Noguchi & Tilden in 1926. Since then, the organism has been used in a number of nutritional studies concerned with the ability of flagellates to synthesize haematin and thiamine (see M. Lwoff, 1951). A. Lwoff (1934) in a study of the role of haematin in the growth and metabolism of three parasitic flagellates found that about 90% of the respiration of *S. oncopelti* could be inhibited by cyanide, while a similar parasite *Strigomonas fasciculata* isolated from the gut of the mosquito *Culex pipiens*, showed a respiration sensitive to cyanide and carbon monoxide, and contained cytochromes *b* and/or *c*.

The present study shows that in its catabolic metabolism, *S. oncopelti* bears a striking resemblance to *Trypanosoma lewisi* (Ryley, 1951). The overall degradation of glucose under both aerobic and anaerobic conditions has been investigated, and the presence of a number of phosphorylating enzyme systems has been demonstrated in cell-free preparations. The flagellate has been found to contain a well-developed cytochrome system, although it seems that some of the respiration is mediated by alternative pathways.

MATERIAL AND METHODS

Organism. The culture of *Strigomonas oncopelti* was kindly supplied by Dr J. Colas Belcour of the Institut Pasteur, Paris; it was the no. 4 strain, originally isolated by Noguchi and Tilden from the latex of *Asclepias syriaca* (M. Lwoff, 1940). The flagellates were cultivated in a sterile medium containing 2% (w/v) 'Oxoid' brand Bacteriological peptone (Oxo Ltd.), 0·6% (w/v) NaCl and 0·8% (w/v) glucose, pH 7·0; incubation was carried out at 24°. Stock cultures were transfered every week, using two drops of culture/tube as inoculum. A second culture line was maintained in a medium devoid of glucose; in this case the flagellates grew, but cultures were not as heavy as in the presence of glucose. Large-scale cultures were grown in 2 l. flasks containing 1·5 l. medium. They were harvested using a centrifuge after 4–6 days' growth, and washed and suspended in a Ringer–phosphate solution containing 0·097 M-NaCl, 0·006 M-KCl, 0·002 M-$MgSO_4$ and 0·025 M phosphate buffer (pH 7·3) or a Ringer–bicarbonate solution containing 0·097 M-NaCl, 0·006 M-KCl, 0·002 M-$MgSO_4$ and 0·016 M-$NaHCO_3$, which when used in conjunction with gas phases containing 5% CO_2 (v/v), had a pH of 7·3. Cell-free preparations (homogenates) were made by washing the organisms once in distilled water, and then shaking a thick aqueous suspension of cells with grade 11 Ballotini glass beads for 5 min. in a Mickle disintegrator.

Materials. Cozymase was prepared from baker's yeast to the 30% purity stage, as described by LePage (1949). Adenosine triphosphate (ATP) was prepared from rabbit muscle as the Ba salt (98–99% pure) by the method of LePage (1945), using two precipitations with Lohmann's reagent, and converted into the Na salt before use. Cytochrome *c* was prepared from ox heart by the method of Keilin & Hartree (1937) as a $2{\cdot}15 \times 10^{-4}$ M solution in 0·9% (w/v) NaCl. Ca fructose 1:6-diphosphate and K glucose 1-phosphate were obtained from L. Light and Co. Ltd.

Spectroscopic observations. These were made using a hand spectroscope (Browning, London) illuminated by a lens system and a pointolite lamp, or a Beck Hartridge reversion spectroscope. The hand spectroscope, although of the prism type, was fitted with a linear scale; this was roughly calibrated with cytochrome *c* and cells having known absorption bands. The more prominent bands of the spectrum were also observed with the reversion spectroscope, which gives wavelength readings direct. Observations were made both at room temperature and after cooling a cell suspension in 50% (v/v) aqueous glycerol with liquid nitrogen (Keilin & Hartree, 1949, 1950).

Metabolic experiments. These were carried out at 30° in Warburg manometers using cups (vol. 20–25 ml.) fitted with two side arms. Gas phases of air or 5% CO_2–95% N_2 (v/v) were used. Respiratory quotients were measured by the second method of Dickens & Šimer (Dixon, 1934), using a Ringer–bicarbonate solution in conjunction with a gas phase of air containing 5% (v/v) CO_2. Metabolic rates were based on dry weight determinations, being expressed as Q_{O_2} or $Q_{CO_2}^{N_2}$, i.e. as μl. gas/mg. dry weight/hr., or on the N content of the suspension, being expressed as q_{O_2} or $q_{CO_2}^{N_2}$, i.e. as μl. gas/mg. N/hr. It was found from a number of determinations that 1 mg. cell N was equivalent to 8·26 mg. dry weight.

Estimation of metabolites. Glycogen was estimated by the method of Good, Kramer & Somogyi (1933). Using $Ba(OH)_2$–$ZnSO_4$ filtrates, reducing sugar was estimated iodometrically by the method of Somogyi (1945), lactic acid by the method of Barker & Summerson (1941), ethanol by distillation and reduction of dichromate as described by Nicloux, le Breton & Doutcheff (1934), and glycerol by the periodate method of Lambert & Neish (1950), slightly modified. For this, the sample was made to 1·5 ml. with

* Part 3, Ryley (1952).

water, 0·5 ml. 2N-H_2SO_4 was added, followed by 0·5 ml. 0·1M $NaIO_4$. Exactly 5 min. later, 0·5 ml. 13·13% (w/v) $NaAsO_2$ was added, and after standing for 10 min., the mixture was diluted to 10 ml. with water. A 1 ml. sample of this was taken and heated with 10 ml. chromotropic acid reagent (1·08 g. Na salt of chromotropic acid in 100 ml. distilled water + 450 ml. 65% (v/v) H_2SO_4) on a boiling-water bath for 30 min. The tubes were cooled, and the colour measured in the Hilger Spekker absorptiometer using an Ilford 606 yellow filter (peak transmittance 580 mμ.) and a 1 cm. cell. With each set of estimations, a series of nine standards was included, containing 0, 50 and 100 μg. glycerol plus 0, 0·5 or 1·0 mg. glucose. During the 5 min. period of the reaction, glucose is slightly degraded by periodate, and from the above standards, it was possible to apply a correction to the glycerol value after the glucose in the metabolic solution had been determined. Using trichloroacetic acid (TCA) filtrates, pyruvic acid was estimated by the method of Friedemann & Haugen (1943), fructose by the method of Roe (1934) and phosphate fractions by the method of Fiske & Subbarow (1925); total phosphorus was estimated after digestion with 60% $HClO_4$ and a little HNO_3. P_0 represents inorganic phosphate estimated directly, P_7 and P_{180} represent inorganic phosphate estimated after hydrolysis in N-HCl at 100° for 7 and 180 min. respectively, while P_t represents the total phosphate found after the $HClO_4$–HNO_3 digestion. Volatile acids were estimated by distillation and titration of a tungstic acid filtrate as described by Buchanan, Sakami, Gurin & Wilson (1947); acid obtained by distillation in the presence of HgO was considered to be acetic, while acid recovered by distillation in the absence of HgO was considered to represent acetic + formic. Succinic acid was estimated manometrically by means of a washed pig-heart preparation of succinic oxidase.

RESULTS

Aerobic metabolism

Respiratory activity. Washed suspensions of *Strigomonas oncopelti* in the absence of extracellular nutrients respire and maintain motility for long periods; from a series of thirteen experiments, a q_{O_2} of 146 (range 103–236) was obtained ($Q_{O_2} = 17·7$). Estimations of intracellular glycogen gave values of 2·48 and 2·80% (on a dry-weight basis) for two 5-day cultures. It is interesting to note that large amounts of soap were recovered after alkaline hydrolysis of the cells in the glycogen determinations. Experiments carried out in a Ringer–bicarbonate medium showed that this endogenous respiration did not involve the formation of acid, and took place with a respiratory quotient (R.Q.) of 0·9 (range 0·87–0·93).

On the addition of extracellular substrate, the respiration of the flagellate can be stimulated as much as fourfold. The twenty-three substances examined (at a concentration of 0·02M) were divided into six groups: (*a*) Glucose, mannose and fructose stimulated respiration by 150–300%, the actual stimulation varying from experiment to experiment. (*b*) L-Glutamate, glutamine, succinate and α-oxoglutarate stimulated respiration by 100–150%. (*c*) DL-Aspartate, asparagine and lactate stimulated respiration by 50–100%. (*d*) Ethanol DL-alanine, glycerol, lactose, sucrose, maltose, and galactose gave respiratory stimulations of up to 50%. (*e*) Citrate, pyruvate, formate and butyrate were without effect on respiration. (*f*) Acetate and propionate inhibited respiration by approximately 20%.

Effect of inhibitors on respiration and motility. The sensitivity of endogenous and glucose-stimulated respiration to a number of inhibitors was investigated; the results are summarized in Table 1. The experiments with cyanide were carried out using balanced KOH–KCN mixtures in the centre well (Robbie, 1946). Cyanide did not exert any appreciable effect on motility until concentrations approaching 0·01M were used; at this concentration, especially in the absence of glucose, the flagellates became sluggish, showing a tendency to round off. On the whole, respiration of the flagellates was more sensitive to inhibitors than was their motility; in general, a large respiratory inhibition was usually accompanied by a small decrease in motility, with some rounding off of the cells, but only in the case of 0·01M arsenite and the higher concentrations of 2:4-dinitrophenol, was complete immobilization of the cells obtained.

Table 1. *Effect of inhibitors on respiration*

Each manometer cup contained about 1 mg. cellular N in a total vol. of 1·5 ml.; gas phase air; pH 7·3 unless otherwise indicated; glucose present where shown in concentration of 0·02M; % inhibition estimated over a 1 hr. period of respiration.

Inhibitor	Concentration (M)	Inhibition (%) No glucose	Inhibition (%) Glucose
Potassium cyanide	$0·46 \times 10^{-3}$	76	88
	10^{-4}	—	65
	$0·46 \times 10^{-4}$	46	37
	$0·46 \times 10^{-5}$	—	18
Hydroxylamine	0·01	54	66
Sodium arsenite	0·01	—	79
	0·003	16	60
	0·001	—	24
Iodoacetate	0·001	50	55
Sodium fluoride	0·04	—	0
Sodium malonate	0·04	−6	0
Phenylthiourea	0·001	—	−5
2:2′-Dipyridyl	0·001	−3	11
8-Hydroxyquinoline	0·001	−7	17
		pH 5·5; glucose	pH 6·2; glucose
Sodium azide	0·0003	81	79
	0·0001	67	33
2:4-Dinitrophenol	0·003	96	77
	0·001	75	51
	0·0003	75	25
	0·0001	21	2

Haem compounds

Spectroscopic observations. Direct spectroscopic examination of a well aerated suspension of the flagellates revealed no absorption bands; there was no indication of the presence of haemoglobin in the organism. On allowing the suspension to stand, absorption bands due to cytochrome pigments became apparent. Spectroscopic examination at room temperature of a cell suspension reduced with a little sodium dithionite ($Na_2S_2O_4$) showed: (*a*) A strong band in the green, having its centre at 558 mμ., and extending over the range 552–564 mμ. This band was probably due to a modified *b* component and/or an *c* component. (*b*) A weaker β-band at 528–530 mμ. (*c*) An extremely weak band due to cytochrome *a* at 605 mμ. Examination of a cell suspension in 50 % glycerol after reduction, cooling in liquid nitrogen, devitrification and recooling, revealed a sharp, fine cytochrome *a* band, and in addition, a sharp and quite intense band due to cytochrome *c*, well separated from the intense band in the green; placing a tube of frozen cytochrome *c* in the light path did not displace, but merely intensified the *c* band of the organism.

Manometric observations. A homogenate of *S. oncopelti* prepared in the Mickle disintegrator was able to catalyse the oxidation of *p*-phenylenediamine, succinate, lactate (Fig. 1), and also *p*-aminophenol. The oxidation of succinate and lactate was slightly stimulated by added cytochrome *c*, but this had no effect on the oxidation of *p*-phenylenediamine. However, if the homogenate was centrifuged, and the deposit washed and resuspended in Ringer–phosphate, the suspension would oxidize *p*-phenylenediamine (q_{O_2} 112), and the rate was stimulated 52 % by $6{\cdot}5 \times 10^{-5}$ M cytochrome *c*. In connexion with the presence of cytochrome oxidase, the inhibitory effects of cyanide shown in Table 1 should be noted. Neither whole cells nor homogenates were able to decompose H_2O_2, indicating the absence of catalases. The flagellates were rapidly immobilized by 0·005 M-H_2O_2.

Anaerobic metabolism

Under anaerobic conditions in the absence of extracellular substrates the flagellates are metabolically inactive, and over a period of about 30 min., gradually lose their motility. In the presence of glucose, fructose or mannose, but not with other mono- or di-saccharides tested, the cells show vigorous motility, and liberate carbon dioxide when suspended in a Ringer–bicarbonate medium; $q^{N_2}_{CO_2}$ values up to 800 have been observed. This evolution of carbon dioxide is due to acid production, plus the balance of carbon dioxide formed by fermentation, and that assimilated. The sensitivity of this carbon dioxide production to a number of inhibitors was tested; the results are summarized in Table 2. From this, it can be seen that the fermentation was little affected by cyanide, azide fluoride or phenylthiourea, was inhibited by rather high concentrations of arsenite or iodoacetate, while a quite large inhibition was obtained with 0·001 M 2:2′-dipyridyl or 8-hydroxyquinoline.

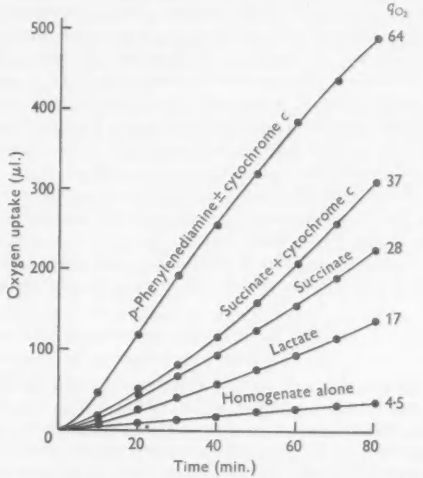

Fig. 1. Oxidase activity in *S. oncopelti* homogenate. Each manometer cup contained 6·4 mg. homogenate N and 0·025 M phosphate buffer in a total vol. of 2·5 ml.; gas phase CO_2-free air. Succinate or lactate tipped from side bulb to give conc. of 0·03 M; *p*-phenylenediamine added at rate of 13 mg./cup; cytochrome *c* when present in conc. of $6{\cdot}5 \times 10^{-5}$ M. Oxygen uptake of *p*-phenylenediamine + cyt. *c* without homogenate over 80 min. period 9 µl.; q_{O_2} of system containing lactate + cyt. *c* (not shown) 20.

Table 2. *Effect of inhibitors on anaerobic metabolism*

Each manometer cup contained about 0·5 mg. cellular N in a total vol. of 1·5 ml.; glucose present at concentration of 0·02 M; gas phase 5 % CO_2–95 % N_2. Inhibitor added from side bulb, and inhibition of CO_2 production estimated over period of 1 hr.

Inhibitor	Concn. (M)	Inhibition (%)
Potassium cyanide	0·01	0
Sodium azide	0·04	41
	0·01	26
Sodium arsenite	0·02	46
	0·01	26
Iodoacetate	0·01	79
	0·002	47
	0·001	25
Sodium fluoride	0·02	19
Phenylthiourea	0·001	0
2:2′-Dipyridyl	0·001	81
8-Hydroxyquinoline	0·001	66

Glucose metabolism

Although endogenous respiration of *S. oncopelti* takes place with a respiratory quotient of 0·9, much higher values were obtained for respiration in the presence of glucose. Using the method of Dickens & Šimer with cells in a Ringer–bicarbonate medium, R.Q.'s up to 2·4 have been obtained; in general young cultures (3–4 days) showed a high R.Q., while older cultures (6–7 days) gave R.Q.'s just greater than unity. Cultures of the flagellate grown in the presence of glucose smell remarkably like yeast; examination of metabolic solutions showed that glucose was fermented to a mixture of organic acids, mainly succinic and pyruvic, together with appreciable quantities of ethanol and glycerol.

Conditions affecting the rate of glucose utilization by the flagellate were investigated in washed suspensions. From Table 3 it can be seen that glucose utilization under both aerobic and anaerobic conditions was stimulated by carbon dioxide, and that in the presence of carbon dioxide, more glucose was destroyed anaerobically than aerobically; carbon dioxide stimulated the production of succinic acid, especially under anaerobic conditions.

Using the method of Dickens & Šimer, it was shown that the only gas produced by anaerobic glucose metabolism in a Ringer–bicarbonate medium, was carbon dioxide. Similar experiments with *Trypanosoma lewisi* and *Tetrahymena pyriformis* (GL) gave the same result, but in the case of *Trichomonas foetus* (Ryley, 1955), another gas, doubtless hydrogen, was produced in addition to carbon dioxide. The products of glucose metabolism under both aerobic and anaerobic conditions were examined in the case of cells in carbon dioxide–bicarbonate systems. Although the actual yields of the various metabolites were variable from experiment to experiment, owing to harvesting the cells at different times in the growth cycle, the same overall picture was obtained. A typical series of results is summarized in Table 4, from which it can be seen that most of the acid was accounted for by pyruvic and succinic acids, and that appreciable amounts of ethanol, and also glycerol were produced; there was always more glycerol and less succinic acid produced under aerobic than anaerobic conditions. On some occasions the yields of ethanol were smaller, and those of succinate larger; when this difference was sufficiently pronounced, there was a net fixation rather than a production of carbon dioxide. Although the amounts of metabolic solution available in the above experiments were not large enough to allow the simultaneous estimation of volatile acids, similar experiments showed that not more than 0·1 mole volatile acid, and usually less was produced per mole glucose degraded under either aerobic or anaerobic conditions.

Table 3. *Effect of environment on glucose metabolism*

Each manometer cup contained 0·68 mg. cellular N in a total vol. of 1·5 ml.; phosphate or bicarbonate buffer as appropriate; glucose (15 μmoles) tipped from side bulb at beginning, and fermentation stopped after 40 min. incubation by 0·2 ml. 2N-H_2SO_4 tipped from second side bulb.

System	Glucose used (μmoles)	Succinic acid formed (μmoles)	Total acid formed (μequiv.)
N_2	4·98	0·63	—
N_2 + 0·02M pyruvate	5·35	0·64	—
N_2 + 5% CO_2	10·15	2·31	12·37
CO_2-free air	5·49	0·00	—
Air + 5% Co_2	8·26	0·50	3·97

Table 4. *Metabolic products formed during aerobic and anaerobic glucose breakdown*

Cellular N (5·16 mg.) in total vol. of 6·0 ml. Ringer–bicarbonate distributed in 3 Dickens & Šimer or 4 Warburg manometer flasks; glucose initially present 90 μmoles; gas phase air or N_2, both containing 5% CO_2. Fermentation stopped after 20 min. with acid, flask contents neutralized, and samples taken for estimation of various metabolites. Aerobically 39·35 μmoles glucose and 15·1 μmoles O_2 used with the formation of 39·6 μequiv. acid. Anaerobically 47·6 μmoles glucose used with the production of 49·0 μequiv. acid. Yields expressed in terms of moles metabolite found/mole glucose disappearing, from which values for carbon and acid recoveries have been calculated. Redox values have been calculated by the method of Johnson, Peterson & Fred (1931); the substrate glucose has a redox value of 0.

	Aerobically			Anaerobically				
							Redox values	
Metabolite	Moles	C atoms (g. atoms)	Acid equiv. (g. equiv.)	Moles	C atoms (g. atoms)	Acid equiv. (g. equiv.)	(−)	(+)
Carbon dioxide	0·95	0·95	—	0·46	0·46	—	0·92	—
Ethanol	0·91	1·82	—	0·58	1·16	—	—	1·16
Glycerol	0·32	0·96	—	0·16	0·48	—	—	0·16
Lactic acid	0·01	0·03	0·01	0·02	0·05	0·02	0	0
Pyruvic acid	0·28	0·84	0·28	0·21	0·63	0·21	0·21	—
Succinic acid	0·22	0·88	0·44	0·43	1·72	0·86	0·43	—
Sum	—	5·48	0·73	—	4·51	1·09	1·56	1·32
C atoms supplied	—	6·00	—	—	6·00	—	—	—
Acid yield (from bicarbonate used)	—	—	1·01	—	—	1·08	—	—

Intermediary glucose metabolism

Using homogenates of the flagellates prepared in the Mickle disintegrator, experiments were carried out to demonstrate the presence of several phosphorylating and dehydrogenase systems. In view of the marked inhibition of anaerobic metabolism (Table 2) produced by 2:2′-dipyridyl and 8-hydroxyquinoline, attempts were made to discover the point of attack of these metal-binding substances. In agreement with the low respiratory stimulation produced by maltose, and the low glycogen content of the cells, no maltase, amylase or phosphorylase activity could be demonstrated in homogenates.

Hexokinase. Hexokinase activity was demonstrated by the liberation of carbon dioxide from a bicarbonate buffer in the presence of glucose and ATP. From Table 5 it can be seen that 44% of the glucose initially present in the system was esterified, 42% of the sugar phosphate being recovered as phosphofructose, while some of the ATP was hydrolysed, both in the presence and in the absence of glucose, with the liberation of inorganic phosphate. The rate of the hexokinase reaction was not influenced by 0·005 M 2:2′-dipyridyl; on adding dipyridyl to the homogenate, a deep pink colour was produced, indicating the presence of ferrous iron.

Phosphoglucomutase and ketoisomerase. The ability of *S. oncopelti* to transform glucose 1-phosphate into a mixture of hexose 6-phosphates has been shown directly by incubating Cori ester with a homogenate of the flagellate; evidence for this is presented in Table 6.

Fructose diphosphate metabolism. Aldolase activity, by which fructose diphosphate (FDP) is converted into triose phosphate (alkali-labile phosphate), was demonstrated in homogenates of *S. oncopelti* at pH 6·0. As can be seen from Table 7, the reaction rate was little influenced by the presence of 0·06 M-KCN and/or 0·005 M 2:2′-dipyridyl, although in the presence of cyanide, which acts as a keto-fixative, more FDP was eventually degraded to triose phosphate.

Fig. 2 illustrates an experiment in which an attempt was made to show the coupling of the oxidation of triose phosphate with the reduction of pyruvate. Fluoride was added to inhibit enolase, and thus cause an accumulation of phosphoglyceric acid; the overall reaction should result in the production of acid, indicated by the liberation of carbon dioxide from a bicarbonate buffer. From the curve it can be seen that the homogenate showed an endogenous fermentation of unknown nature, and that the oxido-reduction was dependent on the presence of added cozymase, was inhibited by 0·001 M iodoacetate, and was stimulated by 0·004 M arsenate. The omission of pyruvate from the system had no effect; lactic acid analyses carried out at the end of the experiment showed that none of this substance was formed during the oxido-reduction. The analyses for fructose and phosphorus indicate that the utilization of FDP paralleled the production of acid, and that in the presence of iodoacetate or in the absence of cozymase, most of the FDP utilized was recovered in the form of triose phosphate, while with the complete system, with or without arsenate, most of the FDP was degraded beyond the triose phosphate stage. At the end of the experiment, the remainder of the TCA filtrate was fractionated to give the barium-insoluble fraction. Although fructose analyses on this fraction showed that much had been lost, there was found to be an accumulation of phosphoglyceric acid in the complete system, and a larger accumulation in the presence of arsenate; this was shown by an increase in phosphate resistant to hydrolysis for 6 hr. in N-HCl, and by the colorimetric method of Rapoport (1937), which gave only a qualitative result as no standard of phosphoglyceric acid was available. The

Table 5. *Hexokinase activity in* S. oncopelti *homogenate*

Each manometer cup contained 1·8 mg. homogenate N, 0·06 M-NaF, 0·005 M-$MgSO_4$ and 0·025 M-$NaHCO_3$ in vol. of 2·4 ml.; gas phase 5% CO_2–95% N_2. ATP (10·75 μmoles) and glucose (11·1 μmoles) added at beginning of experiments and reaction stopped at the end by 0·3 ml. 2 N-H_2SO_4. Manometer contents neutralized; glucose estimated in $Ba(OH)_2$–$ZnSO_4$ filtrates, and fructose and acid-labile phosphate in TCA filtrates. Figures represent μmoles.

	Glucose	No glucose
Glucose used	4·85	—
ATP used [P_7–P_0]	5·49	2·96
Ester-P formed	3·49	0·22
P_0 liberated	2·00	2·74
Fructose 6-phosphate formed	1·46	0·00
CO_2 evolved	6·52	3·40

Table 6. *Phosphoglucomutase and ketoisomerase activity in* S. oncopelti *homogenates*

Each tube contained 3·2 mg. homogenate N, 0·002 M $MgCl_2$, 0·02 M acetate–veronal buffer (pH 7·3) and 3 mg. K glucose 1-phosphate (G 1-P) in a total vol. of 4·5 ml. Tubes deproteinized with 1·5 ml. 10% TCA after 10 or 60 min. incubation at 30°. Figures represent changes in various phosphate fractions expressed in μmoles. Fructose 6-phosphate (F 6-P) from fructose content of TCA filtrate.

Time (min.)	[P_0]	[P_7–P_0] (G 1-P)	[P_{180}–P_7]	[P_f–P_{180}]	F 6-P
10	+1·38	−2·30	+0·40	+0·52	+0·28
60	+2·70	−6·40	+0·75	+2·95	+2·03

Table 7. *Aldolase activity in* S. oncopelti *homogenates*

Tubes were set up to contain 2·2 mg. homogenate N, 0·011 M acetate–veronal buffer pH 6·0, 0·06 M-NaF, 0·004 M fructose 1:6-diphosphate (FDP) and 0·06 M-KCN and 0·005 M 2:2′-dipyridyl where indicated in total vol. of 10 ml. Samples (2 ml.) removed into 2 ml. 7·7% TCA after incubation at 30° for various intervals. Figures represent μmoles FDP used (from fructose estimations) or triose phosphate (alkali-labile) formed.

	Time (min.)	FDP used		Triose P formed	
		Control	2:2′-Dipyridyl	Control	2:2′-Dipyridyl
No KCN	2	4·90	4·90	8·56	6·90
	4	6·60	6·20	11·23	11·03
	10	6·90	7·35	11·80	12·82
KCN	2	4·25	4·50	5·14	5·84
	4	6·40	6·20	9·36	9·51
	10	11·10	10·70	18·16	17·69

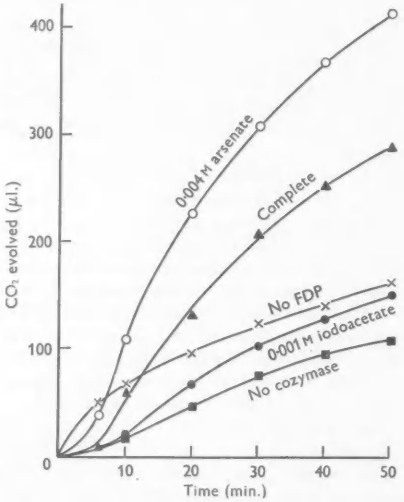

Fig. 2. Fructose diphosphate metabolism by *S. oncopelti* homogenate. Each curve represents CO_2 evolution from 0·025 M-$NaHCO_3$ containing 0·008 M FDP, 0·06 M-NaF, 2 mg. impure cozymase, 0·008 M pyruvate and 8·4 mg. homogenate N in a total vol. of 5·2 ml. distributed between two manometers; gas phase 5% CO_2–95% N_2. Presence of 0·004 M sodium arsenate or 0·001 M iodoacetate as indicated. Reaction started by adding FDP from side bulb; at end, 1·5 ml. 10% TCA added to each cup, and supernatant after centrifuging assayed for fructose and various phosphorus fractions.

System	FDP used (μmoles)	Triose P formed (μmoles)	FDP fermented (μmoles)
Complete	16·35	11·70	10·50
No cozymase	7·60	15·02	0·09
0·001 M Iodoacetate	8·75	14·50	1·50
0·004 M Arsenate	24·78	5·18	22·19

rate of the oxido-reduction reaction, as measured by acid production, was consistently found to be inhibited 31% by the presence of 0·005 M 2:2′-dipyridyl; this inhibition remained unchanged with twice the concentration of dipyridyl.

Carboxylase. It was found that homogenates of *S. oncopelti* in phosphate buffer, pH 6·8, were able to degrade pyruvate with the formation of carbon dioxide. The reaction gave the theoretical yield of carbon dioxide in the presence of a nitrogen gas phase; with a gas phase of air, the carbon dioxide yield was reduced to 87%. The rate of pyruvate breakdown was slightly stimulated by preincubation of the homogenate with ATP and thiamine.

Dehydrogenases. Homogenates of the flagellate were earlier shown to oxidize lactate and succinate (Fig. 1). The ability of such homogenates to oxidize these and some other substrates using methylene blue as hydrogen acceptor has been tested by the Thunberg technique. From Table 8 it can be seen that lactate and fumarate considerably accelerated the reduction of methylene blue, while succinate and ethanol caused a slight stimulation of the reduction; formate, FDP and glucose were without effect. The presence of lactic and succinic dehydrogenases, and fumarase + malic dehydrogenase was also demonstrated by the manometric technique of Quastel & Wheatley (1938), using ferricyanide as hydrogen acceptor. In this system, as can be seen from Fig. 3, the activity of lactic and succinic dehydrogenases was marked, while fumarase + malic dehydrogenase activity was rather low.

DISCUSSION

In contrast to the animal trypanosomes, *Strigomonas oncopelti* is able to maintain respiration and motility for some time in the absence of extracellular substrates. Although the flagellate contains a small amount of material estimated as glycogen, the fact that endogenous respiration takes place with an R.Q. of 0·9, and does not involve the production of acid suggests that some other endogenous substrate is being utilized. A similar situation exists with the ciliate *Tetrahymena pyriformis* (Ryley, 1952) where, although the cells contain about 20% of glycogen, these reserves are only

metabolized under anaerobic conditions, some other intracellular substrate being responsible for endogenous respiration. With the flagellate *Trichomonas foetus*, on the other hand (Ryley, 1955), both aerobic and anaerobic endogenous metabolism depend on intracellular glycogen. A number of substances, particularly hexoses, are able to stimulate the respiration of *S. oncopelti* considerably, although motility does not increase perceptibly. Succinate more than doubles respiration; this may be compared with the flagellate *Trypanosoma lewisi* (Ryley, 1951) which, although it produces succinate during anaerobic glucose metabolism, is unable to oxidize added succinate, unless the cell membrane is destroyed.

Table 8. *Dehydrogenase activity in* S. oncopelti *homogenates*

Thunberg tubes contained 4 ml. homogenate (N content as indicated) in 0·024 M phosphate buffer (pH 7·3). Tubes evacuated and washed with pure N_2 4 times. Methylene blue (0·134 μmoles) and 75 μmoles substrate as indicated added from hollow stopper. Figures give time (min.) for 90% decolorization.

Substrate	Prep. 1 (4·4 mg. N)	Prep. 2 (3·3 mg. N)
None	15	17
Lactate	2	3
Succinate	5·5	9·5
Fumarate	3·5	3·5
Ethanol	10	10·5
Formate	13	—
FDP	20	20
Glucose	20	18

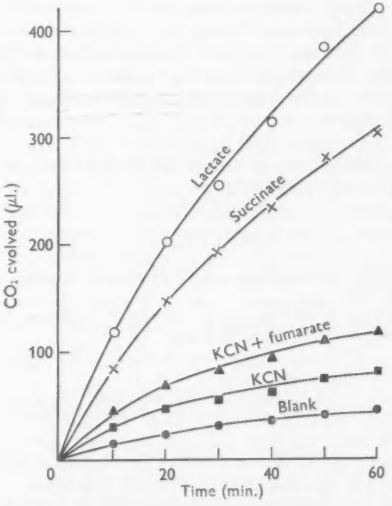

Fig. 3. Dehydrogenase activity of *S. oncopelti* homogenate. Each manometer cup contained 0·02 M-$NaHCO_3$, 0·2 ml. 11% (w/v) $K_3Fe(CN)_6$, 0·08 M succinate, lactate or fumarate and 1·6 mg. homogenate N in a total vol. of 2·5 ml.; in addition, two of the manometers contained 0·05 M-KCN, 0·03 M nicotinamide and 0·4 mg. crude cozymase as indicated; gas phase 5% CO_2–95% N_2.

Spectroscopic examination of *S. oncopelti* reveals the presence of a cytochrome system very similar to that of *T. lewisi*. A. Lwoff (1934) examined the flagellate *S. fasciculata*, but only found absorption bands at 555 and 530 mμ.; it seems likely that the methods used were not sensitive enough to detect the fainter *c* and *a* bands whose presence was suggested by inhibitor experiments; thus in the present study, the cytochrome *c* band only became readily visible after cooling the cell suspension in liquid nitrogen. The presence of cytochrome oxidase is confirmed by the manometric experiments illustrated in Fig. 1; the fact that the oxidation of *p*-phenylenediamine was not stimulated by added cytochrome *c* unless the particulate preparation was first washed suggests that the ratios of cytochromes *a* to *c* is greater than for *T. lewisi*. The oxidation of succinate and lactate by homogenates was also stimulated by added cytochrome *c*. Succinic oxidase activity was greater than lactic oxidase activity, although when assayed by either the manometric or Thunberg tube techniques, lactic dehydrogenase activity was found to be greater than succinic dehydrogenase activity. The presence of such an active lactic dehydrogenase is rather interesting, as significant amounts of lactic acid failed to accumulate during glucose fermentation under either aerobic or anaerobic conditions.

In contrast to its aerobic metabolism, motility and metabolism of *S. oncopelti* under anaerobic conditions depends on the presence of utilizable extracellular carbohydrate. Anaerobically glucose is fermented to a mixture of pyruvic and succinic acids, ethanol, glycerol and carbon dioxide. Under aerobic conditions, glucose fermentation is very pronounced, although succinate production is somewhat decreased, and this vigorous fermentation explains the high respiratory quotients observed in the presence of glucose—up to 2·4 with cells harvested early in the growth period. A. Lwoff (1934) observed an R.Q. of 1·0 for *S. oncopelti*, which suggests that he was dealing with much older cells. Succinate production involves the assimilation of carbon dioxide, although the overall fermentation results in a net production of carbon dioxide, and both glucose utilization and succinate production are considerably stimulated by the presence of carbon dioxide in the gas phase. The formation of glycerol and ethanol during the course of fermentation is interesting. Glycerol production among the Protozoa has been reported for *Trypanosoma brucei* (Glowazky, 1937) and for *T. equiperdum* (Reiner, Smythe & Pedlow, 1936) under anaerobic conditions, while ethanol was detected during anaerobic

glucose metabolism by *T. lewisi* (Reiner *et al.* 1936); the amounts of ethanol produced by *T. lewisi*, and also by *Tetrahymena pyriformis* were shown by Ryley (1951, 1952) to be extremely small. Fulton & Stevens (1945) reported that *T. rhodesiense* produced among many other metabolites glycerol and ethanol; their results however were only of a qualitative nature. Preliminary quantitative experiments indicate that glycerol and pyruvic acid are the main products of aerobic or anaerobic glucose metabolism by *T. rhodesiense*.

Anaerobic glucose fermentation by *S. oncopelti* was remarkably resistant to a variety of inhibitors tested, the most effective substance being 2:2′-dipyridyl, which at a concentration of 0·001 M reduced carbon dioxide production by 81 %. Using this inhibitor with cell-free preparations, it was found that the hexokinase and aldolase systems were insensitive, while there was only a 31 % inhibition of triose phosphate fermentation. In this respect, the aldolase of the flagellate resembles the animal rather than the yeast type of enzyme (Warburg & Christian, 1943).

Cell homogenates are able to ferment hexose diphosphate with the production of acid, including phosphoglyceric acid. This reaction takes place in the presence of 0·06 M fluoride, and is independent of the addition of pyruvate. The experiment illustrated in Fig. 2 was carried out in an attempt to demonstrate the oxidation of triose phosphate coupled to the reduction of pyruvate. Since added pyruvate was not necessary to the fermentation, it seems possible that the enolase system of the flagellate is insensitive to fluoride, and formed rather than added pyruvate is utilized in the reoxidation of cozymase. An alternative explanation is that fluoride inhibits the protozoal enolase, and that the observed acid production is due to an oxido-reduction reaction between two moles of triose phosphate, giving rise to a mixture of α-glycerophosphate and 3-phosphoglyceric acid. This idea is supported by the fact that glycerol accumulates during glucose metabolism, and can stimulate endogenous cellular respiration, but it does not explain the stimulation of hexose diphosphate fermentation produced by arsenate. It should be noted that a similar situation exists with *Trichomonas foetus* (Ryley, 1955); this organism can neither produce nor utilize glycerol, but homogenates can ferment hexose diphosphate in the presence of fluoride, no matter whether pyruvate is added or not.

SUMMARY

1. The parasitic flagellate *Strigomonas oncopelti* can respire and maintain motility under aerobic conditions for several hours in the absence of extracellular substrates. This respiration takes place with a q_{O_2} of about 146 at 30° and an R.Q. of 0·9.

2. Respiration of the flagellate is considerably stimulated by glucose, fructose, mannose, L-glutamate, glutamine, succinate, α-oxoglutarate, DL-aspartate, asparagine and lactate while DL-alanine, ethanol, glycerol, galactose, lactose, sucrose and maltose stimulate respiration to a lesser extent. Citrate, pyruvate and short-chain fatty acids either have no effect or produce a small inhibition of respiration.

3. Respiration of *S. oncopelti* is mediated to a large extent by the cytochrome system. Cytochromes *a*, *b* and *c* have been demonstrated spectroscopically, and cytochrome oxidase and succinic oxidase activity has been shown in cell homogenates.

4. Respiration, and to a lesser extent motility, is quite sensitive to cyanide, azide and 2:4-dinitrophenol.

5. Under anaerobic conditions, metabolism and motility depend on the availability of utilizable extracellular carbohydrate; in a Ringer–bicarbonate medium in the presence of glucose, $q_{CO_2}^{N_2}$ values of up to 800 have been observed. This fermentation is appreciably inhibited by 2:2′-dipyridyl.

6. Under both aerobic and anaerobic conditions, glucose is degraded to a mixture of ethanol, glycerol, succinic and pyruvic acids and carbon dioxide.

7. Using homogenates of *S. oncopelti*, the flagellate has been shown to contain hexokinase, phosphoglucomutase and ketoisomerase, aldolase, a triose phosphate oxidizing system, carboxylase, succinic, lactic and malic dehydrogenases and fumarase.

I should like to thank Miss Margaret Mitchell for her invaluable technical assistance.

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Studies on the Metabolism of the Protozoa

5. METABOLISM OF THE PARASITIC FLAGELLATE *TRICHOMONAS FOETUS**

By J. F. RYLEY
Imperial Chemical (Pharmaceuticals) Limited, Biological Laboratories, Morley, Wilmslow, Cheshire

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Trichomonas foetus is a flagellated protozoon parasitic in the reproductive tracts of bovines. It has a venereal transmission, and is one cause of abortion in cattle. It was first obtained in bacteria-free culture by Witte (1933), and since then numerous biochemical observations have been made on the flagellate in culture, but it was not until 1951 that Suzuoki & Suzuoki (1951) carried out an investigation of the parasite in washed suspension. These workers studied particularly the metabolism of added glucose, and the sensitivity of this metabolism to inhibitors. They failed, however, to recognize the importance of the considerable intracellular glycogen reserves in the energy metabolism of the flagellate, and the relationship of this metabolism to that of extracellular substrates. In the present study, attention has been focused on the glycogen reserves of the parasite as the centre of its catabolic activities, and a number of enzyme systems have been demonstrated in cell-free preparations.

MATERIAL AND METHODS

Organisms. The Belfast strain of *Trichomonas foetus*, the McEntegart strain of *T. vaginalis*, and a strain of *T. gallinae* isolated in 1951 by Dr L. P. Joyner from a crop lesion of a pigeon, were kindly supplied by my colleague Mr W. R. Jones. Stock cultures were maintained in the C.P.L.M. medium of Johnson & Trussell (1943). For bulk growth, *T. vaginalis* was cultivated in McEntegart's (1952) medium containing 10% (v/v) horse serum, while *T. foetus* and *T. gallinae* were cultivated in a peptone–lemco medium which contained 1% (w/v) 'Oxoid' brand bacteriological peptone, 0·5% (w/v) 'Oxoid' Lab-lemco (Oxo Ltd.) and 0·5% (w/v) NaCl. The pH was adjusted to 8·4, using phenolphthalein as indicator, and the medium was then boiled for 5 min. to precipitate phosphates. After cooling, the solution was filtered, the pH adjusted to 7·3, and 0·3% (w/v) glucose added. The medium was autoclaved, and after cooling, 7% (v/v) sterile horse serum was added. Amounts (150 ml.) of this medium were inoculated with a tube of culture in C.P.L.M. medium, and after 48 hr. growth at 37°, half the culture was inoculated into a further 1·5 l. of the same medium. After 48 hr. growth, the organisms were harvested with a centrifuge, and washed and suspended in Ringer–phosphate or Ringer–bicarbonate solutions as described by Ryley (1955).

Materials. The glycogen used in the investigations of intermediary metabolism was a purified specimen prepared from *T. foetus* (Manners & Ryley, 1955). Adenosine triphosphate (ATP), cozymase and cytochrome *c* were prepared as described by Ryley (1955), while Ca fructose 1:6-diphosphate and K glucose 1-phosphate were obtained from L. Light and Co. Ltd.

Methods. The methods used were the same as those employed with *Strigomonas oncopelti*, (Ryley, 1955), except that all experiments were carried out at 37°. Metabolic rates were again based on nitrogen or dry-weight determinations. From a number of experiments, it was found that 1 mg. cellular N was equivalent to $0{\cdot}95 \times 10^8$ flagellates, 10·3 mg. dry weight or about 64 mg. wet weight; these relationships are only approximate, due to the variable glycogen content of the organism; it should be noted that Suzuoki & Suzuoki (1951) state that 'the dry weight of 10^7 parasites was about 10 mg.', a relationship in error by a factor of ten. The present studies were carried out over the same period of time as those reported in the previous paper for *S. oncopelti* (Ryley, 1955); this gave a check on the methods employed, which in several cases gave negative results with *T. foetus*.

* Part 4, Ryley (1955).

RESULTS

In the following paragraphs, it will be shown that the energy metabolism of *Trichomonas foetus* centres round its intracellular glycogen reserves, supplemented by any extracellular substrates available. These are degraded under both aerobic and anaerobic conditions to a mixture of succinic and acetic acids, carbon dioxide, and another gas, which has been shown by Suzuoki & Suzuoki (1951) to be hydrogen. The production of hydrogen rather complicates the manometry, and in the section 'Anaerobic metabolism' the gas exchanges calculated as carbon dioxide represent the sum of carbon dioxide produced by fermentation and due to acid formation, and hydrogen. The techniques available during the course of the present study made the simultaneous measurement of oxygen, hydrogen and carbon dioxide changes impossible, and in the section 'Aerobic metabolism', gas exchanges calculated as oxygen uptake represent the sum of oxygen consumed and hydrogen evolved in a system free from carbon dioxide.

Aerobic metabolism

Respiratory activity. Under aerobic conditions, *T. foetus* maintains respiration and motility for considerable periods of time at the expense of its intracellular glycogen; from a series of eighteen experiments, an average 'q_{O_2}' (μl. O_2 consumed/mg. N/hr.) of 176 was obtained (range 113–255). Twenty-two substrates were tested at a final concentration of 0·02 M for their ability to stimulate the respiration of the flagellate in a phosphate buffer; these substances can be divided into five groups: (*a*) Glucose, fructose and mannose stimulated respiration by 75–125 %. (*b*) Galactose and lactose stimulated respiration by 50–75 %. (*c*) Maltose, sucrose, L-glutamate and glutamine stimulated respiration by 10–25 %. (*d*) DL-Aspartate, asparagine, formate, acetate, propionate, butyrate, lactate, citrate and succinate gave respiratory stimulations of up to 10 %. (*e*) Glycerol, ethanol, pyruvate and DL-alanine were without effect on the rate of respiration.

Effect of inhibitors on respiration and motility. A number of compounds were examined for their ability to inhibit respiration and motility. As indicated in Table 1, most of these substances had little inhibitory effect on endogenous respiration, and in many cases produced a small stimulation; on the other hand, many of them were able to reduce, and in some cases, abolish motility. The most effective inhibitor was iodoacetate, but even with a concentration of 0·01 M, 20 % of the endogenous respiration was retained over the first hour; the effect of this compound increased with time of incubation. Arsenite, another sulphydryl-inhibitor, produced a small stimulation rather than an inhibition of respiration; motility however was reduced almost to zero. A similar uncoupling of respiration and motility was produced by azide. Thus 0·01 M azide at pH 5·5 gave a significant stimulation of respiration, while motility was practically abolished; at pH 6·2, the same concentration of azide was without effect on respiration, but had an adverse effect on the motility of the flagellates. At pH 5·5 0·001 M azide produced similar but smaller effects, but was without action at pH 6·2. 2:4-Dinitrophenol at pH 5·5 inhibited both respiration and motility, although at pH 6·2 there was little effect on either process with concentrations as great as 0·003 M. While 0·01 M hydroxylamine inhibited respiration by only 10 %, it produced a marked reduction in motility. However, this same concentration of hydroxylamine produced a 44 % inhibition of respiration in the presence of added glucose, motility again being reduced to a low level.

Haem compounds

Spectroscopic examination of thick suspensions of the flagellate reduced with a little sodium dithionite ($Na_2S_2O_4$), either at room temperature, or after cooling in liquid nitrogen, failed to show any absorption bands. Similarly, examination of a well-aerated suspension of the organism did not reveal any bands due to oxyhaemoglobin.

Spectroscopic evidence suggests that the cytochromes, if present, occur in only minute amounts. Attempts were made to detect cytochrome oxidase, using the oxidation of *p*-phenylenediamine as a

Table 1. *Effect of inhibitors on respiration and motility of* Trichomonas foetus

Each manometer cup contained about 1 mg. flagellate N in a total vol. of 1·5 ml.; inhibitor added from side bulb, and respiration followed for period of 1 hr.; gas phase air; temp. 37°; organisms examined at end of experiment, and motility assessed as 0 (immobile), +, ++ or +++; negative inhibition indicates respiratory stimulation.

Inhibitor	Concentration (M)	Inhibition (%)	Motility
Potassium cyanide	$0·46 \times 10^{-3}$	0	+++
Sodium fluoride	0·04	8	+
Sodium arsenite	0·04	−21	0
	0·01	−15	0
Iodoacetate	0·01	80	0
	0·001	61	0
	0·0001	30	0
Sodium malonate	0·04	−12	+++
Phenylthiourea	0·001	−7	+++
2:2′-Dipyridyl	0·001	1	++
8-Hydroxyquinoline	0·001	−20	++
Hydroxylamine	0·01	10	+
Azide (pH 5·5)	0·01	−32	0
	0·001	−10	+
2:4-Dinitrophenol (pH 5·5)	0·001	12	+

criterion of activity. Thick homogenates of the trichomonad respired with a q_{O_2} of from 6 to 12; on the addition of *p*-phenylenediamine, the oxygen uptake was stimulated by from two- to fourfold. This extra oxygen uptake could not all be accounted for by the spontaneous oxidation of the *p*-phenylenediamine. Added cytochrome *c* did not produce any stimulation of the oxidation.

Both whole cells and homogenates of *T. foetus* decompose hydrogen peroxide. The catalase activity of the organism is gradually destroyed by peroxide; thus when thick homogenates or cell suspensions were incubated with 10 μmoles hydrogen peroxide, the peroxide was completely broken down within five minutes. When, however, thin homogenates or cell suspensions were used, the peroxide breakdown was slower, and oxygen evolution gradually came to a standstill before all the peroxide had been decomposed, indicating that the catalase system had been inactivated; addition of horse-liver catalase to the system liberated the oxygen from the residual peroxide. Although *T. foetus* contains an active catalase, 0·005 M hydrogen peroxide reduced motility considerably, especially in the case of thin cell suspensions; it is interesting to note that hydrogen peroxide has been used for the treatment of *T. foetus* infection in bulls.

In contrast to the marked catalase activity of *T. foetus*, similar experiments carried out with *T. vaginalis* and *T. gallinae* showed that these flagellates contain no catalase; motility of the organisms is readily abolished by peroxide.

Anaerobic metabolism

Under anaerobic conditions, motility of *T. foetus* is maintained at the aerobic level by fermentation of the intracellular glycogen reserves. In a bicarbonate medium, gas evolution takes place with a '$q^{N_2}_{CO_2}$' of about 325; this gas evolution is almost doubled in the presence of glucose or fructose, galactose or lactose have a smaller effect, while maltose stimulates gas production by about 15 %.

Effect of inhibitors. The effect of a number of inhibitors on endogenous anaerobic metabolism was examined. As can be seen from Table 2, the most effective inhibitor was iodoacetate, which gave 88 % inhibition of gas production at a concentration of 0·0003 M; lower concentrations were not very effective until the cells had been metabolizing in the presence of inhibitor for some time. In contrast to the effect on aerobic metabolism, arsenite gave a small inhibition rather than a stimulation of anaerobic metabolism. High concentrations of arsenite, fluoride, and azide, although not reducing fermentation greatly, reduced motility to a low level; cells which became sluggish owing to the presence of effective inhibitors, also tended to become round in shape.

Glycogen metabolism

Glycogen synthesis and utilization by the trichomonads can be followed in washed suspensions. Tables 3 and 4 indicate that washed suspensions of

Table 2. *Effect of inhibitors on anaerobic metabolism and motility*

Each manometer cup contained about 1·3 mg. flagellate N in a total vol. of 1·5 ml.; gas phase 5 % CO_2–95 % N_2. Inhibitor added from side bulb, and endogenous fermentation followed for period of 1 hr. Flagellates examined on slide in air at the end of the experiment, and motility assessed as in Table 1.

Inhibitor	Concentration (M)	Inhibition (%)	Motility
Potassium cyanide	0·01	25	+ + +
Sodium fluoride	0·04	14	+
Sodium arsenite	0·02	22	+
	0·002	22	+ + +
	0·001	8	+ + +
Iodoacetate	0·0003	88	0
Phenylthiourea	0·001	5	+ + +
2:2′-Dipyridyl	0·001	30	+ + +
8-Hydroxyquinoline	0·001	10	+ + +
Sodium azide	0·02	26	+

Table 3. *Glycogen utilization and formation in washed suspensions (aerobic)*

Manometer cups contained 0·53 mg. flagellate N and 22·5 μmoles substrate in total vol. of 1·5 ml.; gas phase CO_2-free air. System incubated for 90 min. and 0·2 ml. 2 N-H_2SO_4 added from side bulb. Reducing sugar and glycogen estimated in manometer contents after neutralization. Oxygen uptakes have not been corrected for hydrogen production.

Substrate	Oxygen used (μmoles)	Sugar used (μmoles hexose equiv.)	Glycogen change (as μmol. glucose)
None	5·54	0·00	−3·26
Glucose	9·87	11·93	+4·79
Maltose	6·70	3·00	−1·34
Galactose	8·57	9·21	+3·51
Lactose	8·54	9·64	+3·22

Table 4. *Glycogen utilization and formation in washed suspension (anaerobic)*

Cell suspension (0·21 mg. N) incubated in Ringer–bicarbonate medium with 22·5 μmoles substrate for period of 80 min.; gas phase 5 % CO_2–95 % N_2. Reaction stopped by adding acid from side bulb, and reducing sugar and glycogen estimated in manometer contents after neutralization.

Substrate	Acid formed (μequiv.)	Sugar used (μmoles hexose equiv.)	Glycogen change (as μmoles glucose)
None	7·78	0·00	−2·40
Glucose	15·74	6·83	+3·78
Fructose	15·52	6·30	+2·55
Maltose	9·06	0·91	−1·31
Galactose	13·03	4·05	+0·56
Lactose	13·48	5·32	+0·08

the flagellate utilize intracellular glycogen under both aerobic and anaerobic conditions. Maltose exerts a slight sparing effect on the glycogen utilization, while in the presence of glucose, fructose, galactose or lactose, although there is an increase in metabolic rate—oxygen uptake or acid formation—there is a net synthesis rather than breakdown of intracellular glycogen.

During the course of its metabolism, *T. foetus* has been shown to produce hydrogen. Hydrogen production can be measured along with carbon dioxide and acid changes under anaerobic conditions using the Dickens & Šimer technique; hydrogen production in a carbon dioxide-free system can also be followed under anaerobic conditions in ordinary Warburg manometers. Table 5 illustrates an experiment in which glycogen fermentation, and the production of hydrogen, carbon dioxide, succinic and acetic acids was followed under anaerobic conditions. It can be seen that there was a greater utilization of glycogen in the presence of carbon dioxide, and that when present, a small amount of carbon dioxide was assimilated. In the absence of carbon dioxide in the gas phase, the yields of succinic acid per mole hexose utilized were lower, while those of hydrogen, carbon dioxide and acetic acid were correspondingly higher. It should be noted that in view of these results, the direct method of Warburg—using two manometers, one with potassium hydroxide in the centre well, to determine hydrogen and carbon dioxide production under a nitrogen gas phase—is not justifiable, and the figure for carbon dioxide formed in the centre line of Table 5 is only an approximation.

Analysis of the suspending medium after a period of aerobic or anaerobic metabolism showed that the main products of glycogen breakdown were acetic and succinic acids; from Table 6 it can be seen that aerobic conditions favoured the production of acetic acid at the expense of succinic acid, while glycogen breakdown was approximately twice as great under anaerobic as under aerobic conditions. The acetic acid was measured by titration after steam distillation in the presence of mercuric oxide; control experiments showed that this procedure destroyed formic acid. Distillation in the absence of

Table 5. *Effect of environment on anaerobic metabolism*

Manometer cups contained 1·24 mg. flagellate N in total vol. of 2·0 ml.; one series utilized Ringer–bicarbonate medium and Dickens & Šimer manometer flasks with a gas phase of 5% CO_2–95% N_2, while other series utilized Ringer–phosphate with Warburg flasks, and a gas phase of pure N_2 with or without KOH in the centre well. Reaction stopped after 210 min. incubation by tipping 0·3 ml. 2N-H_2SO_4 from side bulb; contents of duplicate manometers pooled, and estimations of glycogen, succinic and acetic acids carried out. In series with CO_2–N_2 gas phase, total acid produced (bicarbonate destroyed) 36·0 μequiv.

Gas phase	Glycogen (μmoles glucose equiv.)	H_2 (μmoles)	CO_2 (μmoles)	Succinic acid (μmoles)	Acetic acid (μmoles)
CO_2–N_2	−12·37	+7·24	−0·56	+12·05	+11·11
N_2	−8·45	—	+6·59*	+6·98	+12·47
N_2 (KOH)	−7·48	+8·02	—	+3·43	+11·98

* This figure is calculated from the total pressure change in the manometer, assuming that 8·02 μmoles H_2 had been produced.

Table 6. *Metabolic products formed during aerobic and anaerobic glycogen breakdown*

Flagellate N (8·20 mg.) in total vol. of 6·0 ml. Ringer–bicarbonate distributed in 3 Dickens & Šimer or 4 Warburg manometer flasks; gas phase air or N_2, both containing 5% CO_2. Fermentation stopped after 57 min. by acid, flask contents pooled, neutralized, and samples taken for estimation of various metabolites. Aerobically, total of 10·80 μmoles glycogen (as glucose equiv.) used with the formation of 27·40 μequiv. acid; anaerobically 22·25 μmoles glycogen and 4·02 μmoles of CO_2 used with the production of 55·6 μequiv. acid and 13·30 μmoles H_2. Yields expressed in terms of moles metabolite found/mole glycogen (as glucose) disappearing, from which values for carbon and acid recoveries have been calculated. Redox values have been calculated by the method of Johnson, Peterson & Fred (1931); the substrate glycogen has a redox value of 0.

	Aerobically			Anaerobically				
							Redox values	
Metabolite	Moles	C atoms (g. atoms)	Acid equiv. (g. equiv.)	Moles	C atoms (g. atoms)	Acid equiv. (g. equiv.)	(−)	(+)
Carbon dioxide	??	—	—	−0·18	—	—	—	0·36
Hydrogen	—	—	—	0·60	—	—	—	0·60
Acetic acid	1·70	3·40	1·70	0·71	1·42	0·71	0	0
Succinic acid	0·44	1·76	0·88	0·95	3·80	1·90	0·95	—
Sum	—	**5·16**	**2·58**	—	**5·22**	**2·61**	**0·95**	**0·96**
Acid yield (from bicarbonate used)	—	—	2·53	—	—	2·50	—	—
C atoms supplied	—	6·??	—	—	6·18	—	—	—

mercuric oxide did not give any greater yield of volatile acid, indicating that no formic acid was produced during glycogen metabolism. Similarly, application of the colorimetric method of Grant (1947) to a $Ba(OH)_2$–$ZnSO_4$ filtrate of the metabolic solution failed to detect any formic acid production. Analysis of similar metabolic solutions showed that no detectable amounts of ethanol, glycerol or pyruvic acid were produced, while lactic acid recoveries indicated that not more than 0·05 mole of the acid was produced/mole hexose utilized.

Intermediary carbohydrate metabolism

Using cell-free preparations (homogenates) made in the Mickle disintegrator, a number of phosphorylative enzyme systems were demonstrated in the flagellate. As with *Strigomonas oncopelti*, the sensitivity of a number of these systems to 2:2′-dipyridyl was tested. On centrifuging a homogenate prepared by disintegration of an aqueous cell suspension, a yellowish brown, opalescent supernatant was obtained, along with a very small deposit, which on microscopical examination was found to consist of cell walls, looking like empty shells, along with the axostyles of the trichomonads.

Amylase and maltase. Cell-free preparations of *Trichomonas foetus*, when incubated with starch, glycogen or maltose in the absence of inorganic phosphate, were found to liberate reducing sugar; this is illustrated in Fig. 1. As the only preparation of glucose oxidase available was contaminated with maltase, it was not possible to demonstrate glucose as the end product of starch and glycogen hydrolysis. In view of the hydrolytic activity towards maltose, however, it seems probable that glucose was produced from starch and glycogen.

Phosphorylase. Cell-free preparations of *T. foetus* were found to degrade starch or glycogen in the presence of inorganic phosphate. From Fig. 2 it can be seen that starch was attacked more rapidly than glycogen (observed on several occasions), and that some of the phosphate esterified was recovered in combination with fructose. In this particular experiment, there was a liberation of inorganic phosphate on prolonged incubation.

Hexokinase. Besides utilizing intracellular glycogen in its energy metabolism, *T. foetus* is able to metabolize free hexoses. Homogenates of the flagellate were found to phosphorylate glucose in the presence of ATP; and most of the acid-labile phosphate of the ATP was recovered as esterified phosphate, but a small amount was liberated as inorganic phosphate, especially in the absence of glucose (Table 7).

Phosphoglucomutase and ketoisomerase. Although in the phosphorylase and hexokinase systems, the production of fructose compounds has been demonstrated, the ability of the trichomonad to transform glucose 1-phosphate to less easily hydrolysable phosphate compounds and phospho-

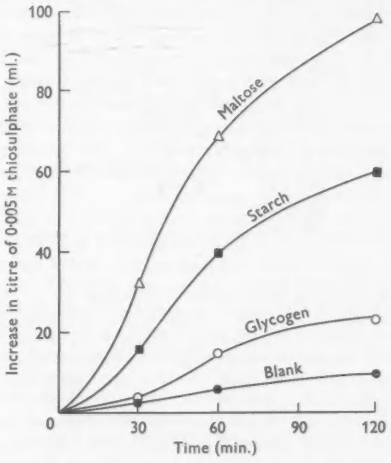

Fig. 1. Carbohydrase activity in *T. foetus* homogenate. Tubes set up to contain 36 mg. glycogen or starch or 68 mg. maltose in 4 ml. 0·036 M acetate–veronal buffer (pH 7·3); 5 ml. homogenate (10 mg. N) added, and samples removed at intervals for estimation of reducing sugar in $Ba(OH)_2$–$ZnSO_4$ filtrates.

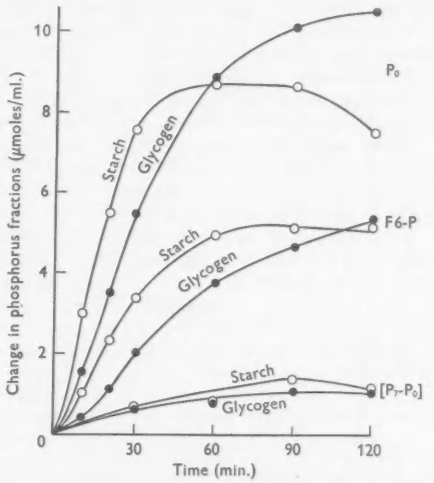

Fig. 2. Phosphorylase activity in *T. foetus* homogenate. Tubes set up to contain 80 mg. starch or glycogen in 2·5 ml. 0·1 M phosphate buffer (pH 7·3). 7·5 ml. homogenate (26·1 mg. N) added and 1 ml. samples removed into 7 ml. 5% TCA after various intervals of incubation at 37°. Fructose, P_0 and P_7 determinations carried out in TCA filtrates.

Table 7. *Hexokinase activity in* T. foetus *homogenate*

Each manometer cup contained 3·5 mg. homogenate N, 0·06 M-NaF, 0·006 M-$MgSO_4$ and 0·025 M-$NaHCO_3$ in vol. of 2·4 ml.; gas phase 5% CO_2–95% N_2. ATP (10·75 μmoles) and glucose (11·1 μmoles) added at beginning of experiment, and reaction stopped at end by 0·3 ml. 2 N-H_2SO_4. Manometer contents neutralized, and glucose estimated in $Ba(OH)_2$–$ZnSO_4$ filtrates, and fructose and acid-labile phosphate in TCA filtrates; results expressed in μmoles.

	Glucose	No glucose
Glucose used	5·60	—
CO_2 evolved	6·20	1·79
ATP used [P_7–P_0]	6·08	2·55
Ester P formed	5·72	0·78
P_0 liberated	0·36	1·77
F 6-P formed	1·97	—

Table 8. *Phosphoglucomutase and ketoisomerase activity in* T. foetus *homogenates*

Each tube contained 0·75 mg. homogenate N, 0·002 M-$MgCl_2$, 0·02 M acetate–veronal buffer (pH 7·5) and 3 mg. K glucose 1-phosphate (G 1-P) in total vol. of 4·5 ml.; tubes deproteinized with 1·5 ml. 10% TCA after 15 or 60 min. incubation at 37°. Figures represent changes in various phosphate fractions expressed in μmoles. Fructose 6-phosphate (F 6-P) from fructose content of TCA filtrate.

Time (min.)	P_0	[P_7–P_0] (G 1-P)	[P_t–P_7]	F 6-P
15	+0·19	−5·04	+4·85	+1·91
60	+0·93	−6·02	+5·09	+1·94

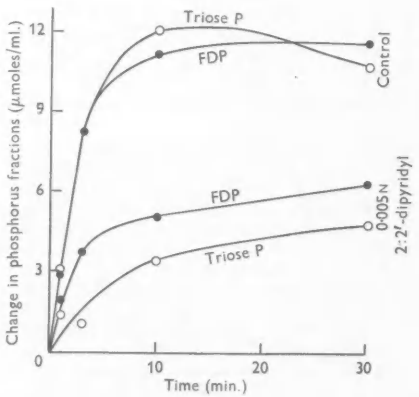

Fig. 3. Aldolase activity in *T. foetus* homogenate. Tubes contained 7·5 mg. homogenate N, 0·01 M acetate–veronal buffer, pH 6·0, 0·06 M-NaF, 0·004 M fructose 1:6-diphosphate (FDP) and 0·005 M 2:2′-dipyridyl where indicated in total vol. of 10 ml. Samples (2 ml.) removed into 2 ml. 7·5% TCA after incubation at 37° for various intervals, and fructose and alkali-labile phosphate estimations carried out in filtrates.

fructose has been shown directly by incubating homogenates with Cori ester; this is recorded in Table 8.

Fructose diphosphate metabolism. Aldolase activity was demonstrated in homogenates of *T. foetus* by incubating them with fructose diphosphate (FDP) and following the liberation of alkali-labile phosphate. This is illustrated by Fig. 3, which shows that approximately 1 mole only of triose phosphate accumulated for each mole of FDP disappearing; this is doubtless due to further degradation of triose phosphate. In the presence of the keto-fixative cyanide, although the initial rate of the reaction was the same, more FDP was eventually converted into triose phosphate. 2:2′-Dipyridyl (0·005 M), either in the presence or in the absence of cyanide, considerably reduced the rate of the aldolase reaction; inhibitions of from 50 to 75% of the initial reaction rate were observed in a number of experiments.

Attempts were made to show the oxidation of triose phosphate coupled to the reduction of pyruvate, the reaction being followed by the pro-

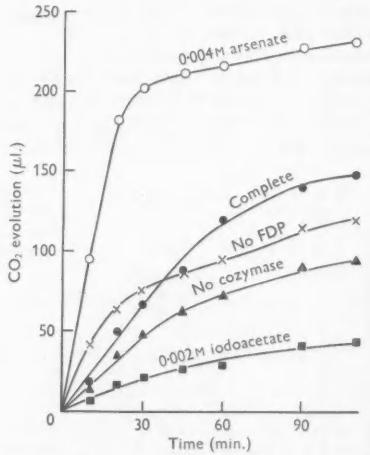

Fig. 4. Fructose diphosphate metabolism by *T. foetus* homogenate. Curves represent CO_2 evolution from 0·025 M-$NaHCO_3$ containing 0·008 M FDP, 0·06 M-NaF, 1 mg. impure cozymase, 0·005 M pyruvate and 3·7 mg. homogenate N in total vol. of 2·5 ml.; gas phase 5% CO_2–95% N_2; 0·004 M sodium arsenate or 0·002 M iodoacetate present as indicated. Fructose and alkali-labile phosphate assayed in TCA filtrates at end:

System	FDP used (μmoles)	Triose P found (μmoles)	FDP fermented (μmoles)
Complete	7·77	3·92	5·81
No cozymase	4·73	5·82	1·82
Arsenate	14·10	2·09	13·06
Iodoacetate	7·85	7·72	3·99

duction of acid. Fig. 4 indicates that the homogenate used showed a considerable endogenous acid production. In the presence of FDP, this acid production was somewhat increased, and FDP was utilized, the greater part being metabolized beyond the triose phosphate stage. This fermentation was not affected by the omission of pyruvate from the system, was much reduced by the omission of cozymase, or by the presence of 0·002 M iodoacetate, and was considerably stimulated by the presence of 0·004 M arsenate; 0·005 M 2:2′-dipyridyl was without effect on the rate of fermentation of FDP.

Dehydrogenases. Attempts were made to detect dehydrogenase activity both by the Thunberg tube technique and manometrically using ferricyanide as hydrogen acceptor. In both cases, however, dehydrogenase activity was marked without the addition of substrates, although lactate very slightly accelerated methylene-blue reduction. Succinate and lactate were on occasions able to increase the oxygen uptake of homogenates, but here again the results were not consistent, owing no doubt to the presence of substrates in the homogenate which were responsible for the appreciable endogenous respiration.

DISCUSSION

During growth, *Trichomonas foetus* synthesizes large amounts of intracellular glycogen. This glycogen is partially derived from the nitrogenous components of the medium, but there is a greater synthesis in the presence of added carbohydrate (Manners & Ryley, 1955). When transferred to a non-nutrient medium, the flagellate utilizes these glycogen reserves with the production of organic acid and hydrogen. The transformation of extracellular simple carbohydrate to intracellular polysaccharide can be demonstrated in washed suspensions of the flagellate; on the addition of glucose, fructose, galactose or lactose, there is an increased production of organic acid, but at the same time a net synthesis of intracellular carbohydrate takes place. In their study of the metabolism of added glucose, Suzuoki & Suzuoki (1951) failed to recognize this simultaneous assimilation, and so their figures for acid yield and oxygen utilization, when expressed in terms of glucose disappearing, are erroneous. They found that glucose fermentation resulted in the production of large amounts of succinic acid, and that only insignificant amounts of lactic and pyruvic acids accumulated. The residue of the organic acid formed during glycogen fermentation has been accounted for as acetic acid; no evidence could be found for the production of formic acid, nor for its utilization by whole cells or homogenates. It seems likely that *T. foetus* contains a phosphoroclastic system similar to that of *Clostridium butyricum* (see Wolfe & O'Kane, 1953), and that pyruvate is broken down to 'active' acetate, hydrogen and carbon dioxide, without the intermediate production of formate, as is the case with *Escherichia coli*. It is of interest to note that Kupferberg, Singher, Lampson, Levy & Romano (1953) find that lactic acid accounts for most of the acid produced during growth of *Trichomonas vaginalis*.

There seems to be some variation between different strains in their ability to metabolize various carbohydrates; thus it is generally contended that *T. foetus* utilizes maltose, Suzuoki & Suzuoki finding that this sugar stimulated respiration by 136%, while Cailleau (1937) found it was fermented during growth. Our strain, however, is only slightly stimulated by maltose, and this sugar is unable to completely replace endogenous glycogen to support normal metabolism, as is the case with glucose, fructose, mannose, galactose or lactose. Our strain also differs from that of Suzuoki & Suzuoki in that it readily utilizes lactose, but neither sucrose nor glycerol.

Riedmüller (1936) found that the respiration of *T. foetus* was insensitive to 95% carbon monoxide in the gas phase, and he was unable to detect any cytochrome pigments, catalase or peroxidase. Likewise Suzuoki & Suzuoki found that respiration was insensitive to cyanide, but Professor K. Okunuki observed absorption bands at 565 and 535 mμ. due to cytochrome *b* in their preparations. In the present study, respiration was found to be insensitive to $0{\cdot}46 \times 10^{-3}$ M cyanide, and spectroscopic examination of thick cell suspensions, even with the use of the liquid air technique, failed to reveal absorption bands. Similar procedures had revealed a well-developed cytochrome system in *Strigomonas oncopelti*, and although it cannot be said that *T. foetus* is devoid of cytochromes, such pigments if present, occur in extremely low concentration, and it would seem that the bulk of cellular respiration is mediated by alternative systems. Cell-free preparations of *Trichomonas foetus* prepared in the Mickle disintegrator did not show any convincing oxidative activity towards *p*-phenylenediamine, suggesting the absence of cytochrome oxidase. Seaman (1954), in reference to similar results previously obtained with *Tetrahymena pyriformis* (Ryley, 1952), has contended that this lack of activity is due to the method of preparing the homogenates, which in his opinion destroys most of the enzyme systems originally present in the cells; Eichel (1954) also disagrees with Seaman concerning the presence of cytochrome oxidase in *T. pyriformis*. It is noteworthy that cytochrome oxidase activity can be readily detected by the present technique in *Trypanosoma lewisi* and *Strigomonas oncopelti* (Ryley, 1951, 1955),

and that although *Trichomonas foetus* homogenates do not show cytochrome oxidase activity, such preparations are far from being metabolically inactive.

Among the enzyme systems which have been demonstrated in homogenates of the trichomonad, the aldolase system is of particular interest. 2:2′-Dipyridyl, in a concentration of 0·005 M inhibited triose phosphate formation by up to 75 %, suggesting that the enzyme system involved requires ferrous iron for activity (see Ryley, 1953). Baernstein (1954) has recently reported that the aldolase system of *T. vaginalis* is sensitive to dipyridyl; he obtained 75 % inhibition of activity with 0·01 M 2:2′-dipyridyl, and found that the inhibition was completely reversed by 10^{-3} M ferrous or 10^{-5} M cobaltous salts. In the case of *T. foetus*, the aldolase system is not rate-limiting in carbohydrate breakdown, even in the presence of dipyridyl, as this inhibitor has little effect on the respiration or fermentation of whole cells, or acid formation from hexose diphosphate by homogenates.

Suzuoki & Suzuoki (1951) state that 'the carbohydrate metabolism of *T. foetus* seems to bear rather striking similarities to that of the African pathogenic trypanosomes', a fact which they suggest should help in the search for potential anti-trichomonad drugs. While it cannot be denied that the respiration of both the pathogenic trypanosomes and trichomonads is independent of cytochrome pigments, it should be noted that with the pathogenic trypanosomes, cyanide usually produces an appreciable respiratory *stimulation* (von Brand & Johnson, 1947). Perhaps the most striking feature in the carbohydrate metabolism of the pathogenic trypanosomes, when compared with more primitive members of the family, is the tendency to lose the ability to degrade carbohydrate beyond the pyruvate stage, equimolecular amounts of glycerol and pyruvic acid being produced under anaerobic conditions and pyruvic acid being the main metabolic product under aerobic conditions. *T. foetus*, on the other hand, carries its carbohydrate metabolism beyond the pyruvate stage, giving rise to succinic and acetic acids; such is the case with the more primitive trypanosomes, e.g. *T. lewisi*. Hydrogen and possibly methane, which are also produced during carbohydrate breakdown by *T. foetus*, are unusual metabolites for protozoa.

SUMMARY

1. The parasitic flagellate *Trichomonas foetus* can maintain metabolism and motility in the absence of extracellular nutrients for considerable periods at 37° under both aerobic and anaerobic conditions; this endogenous metabolism depends on the fermentation of intracellular glycogen.

2. Under aerobic conditions, the parasite respires with a q_{O_2} of about 176 at 37°. This oxygen uptake is doubled in the presence of either glucose, fructose or mannose, and is considerably stimulated by both galactose and lactose, while maltose, sucrose, glutamate and glutamine give small respiratory stimulations.

3. Respiration of the flagellate is probably independent of cytochrome pigments; no evidence for the presence of cytochromes could be obtained by spectroscopic examination, by the use of respiratory inhibitors, or by the use of cell homogenates with *p*-phenylenediamine. *T. foetus* shows vigorous catalatic activity; *T. vaginalis* and *T. gallinae* do not contain catalase.

4. At pH 5·5 0·04 M arsenite or 0·01 M azide appreciably stimulate the endogenous respiration of *T. foetus*, but at the same time abolish cellular motility.

5. Under anaerobic conditions, endogenous metabolism takes place with a $q^{N_2}_{CO_2}$ of 325; this fermentation is 88 % inhibited by 0·0003 M iodoacetate, but is relatively insensitive to other inhibitors tested.

6. Intracellular glycogen disappears on incubation under aerobic or anaerobic conditions in the absence of extracellular substrate; in the presence of glucose, fructose, lactose or galactose, although oxygen uptake or carbon dioxide output is considerably increased, there is a net synthesis of glycogen; maltose exerts a slight sparing action on the utilization of intracellular glycogen.

7. Under aerobic or anaerobic conditions, glycogen is fermented to a mixture of succinic and acetic acids; there is a net formation or assimilation of carbon dioxide depending on the conditions, and a gas other than carbon dioxide is produced. Glycogen breakdown and succinic acid production are favoured by anaerobic conditions and the presence of carbon dioxide in the gas phase.

8. Cell-free preparations contain amylase, maltase, phosphorylase, hexokinase, phosphoglucomutase and ketoisomerase, aldolase and a triose phosphate oxidizing system. The activity of aldolase can be inhibited by as much as 75 % in the presence of 0·005 M 2:2′-dipyridyl.

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Studies on the Metabolism of the Protozoa

6. THE GLYCOGENS OF THE PARASITIC FLAGELLATES *TRICHOMONAS FOETUS* AND *TRICHOMONAS GALLINAE**

By D. J. MANNERS and J. F. RYLEY
Chemistry Department, University of Edinburgh and Imperial Chemical (Pharmaceuticals) Limited, Biological Laboratories, Morley, Wilmslow, Cheshire

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The molecular structures of the intracellular carbohydrates synthesized by a number of Protozoa have been the subject of several recent investigations. Bourne, Stacey & Wilkinson (1950) have examined the polysaccharide synthesized by the flagellate *Polytomella coeca*; it was found to resemble starch, and could be fractionated into linear and branched components (amylose and amylopectin). The polysaccharides produced by the ciliate *Cycloposthium* and by a holotrich ciliate present in the rumen of the sheep differ in that they are homogeneous, and have branched structures similar to that of an amylopectin (Forsyth, Hirst & Oxford, 1953; Forsyth & Hirst, 1953). In contrast, the ciliate *Tetrahymena pyriformis* synthesizes a homogeneous polysaccharide which has the properties of a typical animal glycogen (Manners & Ryley, 1952). During a study of the immunologically specific substances of *Trichomonas foetus*, Feinberg & Morgan (1953) isolated a glycogen-like polysaccharide from dried cells in a yield of 5–10%. The present paper is concerned with the intracellular polysaccharides synthesized by *Trichomonas foetus* and *T. gallinae* (hereafter designated TFP and TGP respectively) which have been purified and subjected to a detailed structural analysis. The available data indicates that they are branched α-1:4-glucosans which resemble glycogen rather than amylopectin in branching characteristics.

MATERIALS AND METHODS

Materials

Flagellate preparations. Trichomonas gallinae and the Belfast strain of *T. foetus* were cultivated in a peptone–lemco medium containing 2% (w/v) glucose as described by Ryley (1955). Cells of *T. gallinae* were harvested after 24 hr. and those of *T. foetus* after 48 hr. incubation at 37° on the centrifuge, and were washed in 0·9% (w/v) NaCl.

Maltulose. Maltulose was prepared from maltose as described by Peat, Roberts & Whelan (1952).

Methods

Before analysis, the polysaccharides were dried *in vacuo* at 100°, over P_2O_5 for several hours.

Blue value (B.V.) was determined by the method of Bourne, Haworth, Macey & Peat (1948).

Paper chromatography. Descending chromatograms were carried out at room temperature using Whatman no. 1 paper and a mixture of 10 ml. glacial acetic acid and 90 ml. of 75% (v/v) aqueous *iso*propanol as solvent (Bird & Hopkins, 1954). Development was by spraying with aniline oxalate (Partridge, 1949), urea oxalate (McGilvray, 1949) and ammoniacal silver nitrate (Trevelyan, Procter & Harrison, 1950). The rate of movement (R_G values) of sugars was determined by dividing the distance moved by the sugars from the starting line by the distance moved by D-glucose ($R_G = 1$) under identical conditions.

α-Amylolysis. α-Amylolysis was investigated by adding salivary amylase solution (5 ml.) to the glycogen (*ca.* 40 mg.) dissolved in 0·2 M phosphate buffer (pH 7·0; 4 ml.), aqueous sodium chloride (3%, w/v; 1 ml.) and distilled water (40 ml.). Samples (2 or 3 ml.) were withdrawn at intervals for determination of reducing power, as maltose. The salivary amylase solution was prepared by dissolving freeze-dried saliva in distilled water, and removing insoluble material with the centrifuge; the solution contained traces of maltotriase but showed no maltase activity.

Sedimentation constant. The rates of sedimentation of the glycogens (1%, w/v, solution in M-NaCl) were determined using an electrically driven 'Spinco' ultracentrifuge (Specialized Instruments Corporation, Belmont, California), as described by Greenwood & Manners (1955). We are indebted to Dr C. T. Greenwood for these determinations.

* Part 5, Ryley (1955).

Other methods. End-group assay, β-amylolysis and other methods were the same as those described previously (Manners & Ryley, 1952), except that reducing sugars were determined using the Shaffer & Somogyi (1933) reagent, as modified by Hanes & Cattle (1938).

RESULTS

Glycogen content of the cells

Trichomonas foetus during growth synthesizes glycogen; the cell glycogen content (dry weight basis) usually lies between 10 and 30 %, although on one occasion a value of 55 % was observed. Growth of the flagellate will take place in the lemco–peptone medium without the addition of glucose; in one experiment such cells had a glycogen content of 8·1 %. In the presence of 1 % glucose, 6 times the yield of organisms was obtained, and these had a glycogen content of 17·4 %. Increasing the glucose content of the medium to 2–6 % reduced growth by half, and gave cells with a glycogen content of about 14 %. Similar values for intracellular glycogen were obtained with *T. gallinae*.

Isolation and purification of glycogen

Cells from 6 to 12 l. batches of culture were treated with 2 vol. 30 % (w/v) KOH at 100° for 30 min., cooled, centrifuged to remove insoluble material, and the crude glycogen was precipitated with 1·1 vol. ethanol. The precipitate was removed on the centrifuge, dissolved in a little water, any insoluble residue centrifuged off, and the glycogen reprecipitated with 1·1 vol. ethanol. The precipitate was washed twice with ethanol, and then with ether, and was air-dried.

The combined preparations of crude glycogen were purified by precipitation with 80 % (v/v) acetic acid (Bell & Young, 1934). Crude glycogen (13 g. TFP; 1·58 g. TGP) was dissolved in water to give a 7 % (w/v) solution, centrifuged to remove any insoluble material, and 4 vol. glacial acetic acid were then added. The precipitated glycogen was recovered by centrifuging and subjected to five more acetic acid precipitations. The glycogen was then 'deacetylated' by treating with 100 ml. 15 % (w/v) NaOH at 100° for 1 hr. (Illingworth, Larner & Cori, 1952). Glycogen was precipitated from the alkaline solution with ethanol, washed in ethanol and ether and air-dried. Yields: TFP 10·13 g., TGP 0·94 g.

Characterization of purified glycogens

The purified glycogens were white powders: TFP, N = 0·04 %, P = nil, ash = 0·2 %; TGP, N = nil, P (inorganic) = 2·30 %, P (organic) = nil, ash = 12·08 %. Weights of TGP are corrected for 12·08 % ash in the material which has no structural significance. The glycogens were freely soluble in water, giving opalescent solutions which stained yellow-brown with dilute iodine (B.V. = 0·01). TFP had $[\alpha]_D^{18} + 199°$ in water (2 dm. tube, *c*, 0·4 or 0·5). TGP had $[\alpha]_D^{18} + 197°$ in water (2 dm. tube, *c*, 0·4 or 0·6).

Glucose content. Hydrolysis of the glycogens by 1·6 N-sulphuric acid at 100° gave the following percentage conversions into glucose: TFP, 96 % in 2 hr., 98 % in 3 hr.; TGP, 82 % in 2 hr., 85 % in 3 hr. Paper chromatography showed the presence of glucose in the hydrolysates; no other reducing sugar was present.

α-Amylolysis. Treatment of the polysaccharides with salivary α-amylase at pH 7·0 and 35° caused a rapid decrease in turbidity and iodine-staining power; the glycogens were achroic within 5 min. Paper chromatography showed the presence of glucose, maltose ($R_G = 0·62$) and α-dextrins ($R_G \leqslant 0·11$) in the α-amylolytic digests. Maltulose ($R_G = 0·66$) was absent. The extent of α-amylolysis (see Table 1) and the presence of α-dextrins in the digest indicates that the glycogens are branched α-1:4-glucosans (cf. Manners, 1954). The slow increase in reducing power after 49 hr. was due to traces of maltotriase in the α-amylase (cf. Manners, 1954). The absence of maltulose is evidence that fructose is not a constituent of the glycogens (cf. Peat *et al.* (1952), who isolated *ca.* 5 % of maltulose from an α-amylolytic digest of pregnant doe-liver glycogen).

End-group assay. Potassium periodate oxidation at room temperature. On complete oxidation, 421 mg. TFP gave 8·11 mg. formic acid, corresponding to a unit-chain length of fifteen glucose residues. 144 mg. TGP under identical conditions gave 4·66 mg. formic acid, corresponding to a unit-chain length of nine glucose residues.

β-Amylolysis. TFP (43·2 mg.) on treatment with crystalline sweet-potato β-amylase (Balls, Thompson & Walden, 1946; Balls, Walden & Thompson, 1948), at 35° and pH 4·54, gave 27·2 mg. maltose, equivalent to a β-amylolysis limit of 60 %. TGP (19·5 mg.), under similar conditions, gave 10·5 mg. maltose, equivalent to a β-amylolysis limit of 51 %.

Sedimentation constants. The ultracentrifugal examination showed that TFP was polydisperse, and contained two components. The major component had $S_{20} = 63 \times 10^{-13}$, and the minor component $S_{20} = 7 \times 10^{-13}$. TGP, although polydisperse, contained only one component which had

Table 1. *Action of salivary α-amylase on glycogens*

The glycogens (0·1 %, w/v) were incubated with salivary α-amylase at 35° in the presence of 0·02 M phosphate buffer and aqueous NaCl (0·075 %, w/v). Samples were analysed at intervals for reducing sugar (as maltose).

Time of incubation (hr.)	Apparent % conversion into maltose	
	TFP	TGP
0·75	30	31
20	58	61
49	61	69
112	63	73

Table 2. *A comparison of the properties of certain protozoal polysaccharides with rabbit liver glycogen and an amylopectin (waxy maize starch)*

Property	Waxy maize* starch	*Cyclo-posthium*†	Holotrich† ciliate	*Tricho-monas foetus*	*Tetra-hymena*‡ *pyriformis*	Rabbit liver§ glycogen	*Tricho-monas gallinae*
$[\alpha]_D$ (water)	+212°	—	—	+199°	+195°	+198°	+197°
$[\alpha]_D$ (N-NaOH)	+153°	+154°	+171°	—	—	+169°	—
Iodine coloration	Red-purple	Purple	Reddish purple	Yellow-brown	Yellow-brown	Reddish brown	Yellow-brown
Unit-chain length							
(*a*) KIO_4 method	—	23–24	22	15	13	13	9
(*b*) Methylation method	22	22	21	—	—	13	—
β-Amylolysis limit	54	—	—	60	44	45	51
Mean exterior chain length‖	14–15	—	—	11–12	8–9	8–9	6–7
Mean interior chain length‖	6–7	—	—	2–3	3–4	3–4	1–2
Approx. mol.wt.							
(*a*) Sedimentation–diffusion	—	—	—	3×10^6	3×10^6	4×10^6	$3–4 \times 10^6$
(*b*) Viscosity of methyl ether	8×10^5	2×10^5	2×10^5	—	—	—	—

Sedimentation–diffusion data taken from Greenwood & Manners (1955).

* Data from Haworth, Hirst & Woolgar (1935), and Halsall, Hirst, Hough & Jones (1949).
† Data from Forsyth *et al.* (1953), and Forsyth & Hirst (1953).
‡ Data from Manners & Ryley (1952).
§ Data from Bell & Manners (1952) and Manners (1952).
‖ Mean exterior chain length = no. of glucose residues removed on β-amylolysis plus 2·5. Mean interior chain length = unit-chain length – (exterior chain length plus 1). Cf. Manners (1954).

$S_{20} = 76 \times 10^{-13}$. Assuming that the diffusion constants were similar to those of other glycogens (see Bell, Gutfreund, Cecil & Ogston, 1948), these sedimentation constants are equivalent to molecular weights of 2·9, 0·3 and $3·5 \times 10^6$ respectively.

DISCUSSION

The present investigation has shown that TFP and TGP superficially resemble other known protozoal polysaccharides in being branched α-1:4-glucosans; they differ, however, from these, and from each other, in degree of branching and in the mean position of branching in the unit-chains, as shown in Table 2. The major proportion of the molecules of TFP are composed of *ca.* 1200 unit-chains, each comprising an average of 15 α-1:4-linked glucose residues, whereas those of TGP contain *ca.* 2000 unit-chains, consisting of only 9 α-1:4-linked glucose residues. TGP thus has a higher degree of branching than that of any other protozoal polysaccharide hitherto described. These differences in degree of branching presumably reflect differences in the enzyme system catalysing the synthesis of glycogen; in *T. gallinae* the ratio of the activity of branching enzyme:phosphorylase must be greater than in *T. foetus* and in *Tetrahymena pyriformis*. The β-amylolysis limits of TFP and TGP are *ca.* 10% higher than those of animal glycogens of similar chain length (cf. Bell & Manners, 1952); hence the ratio of the mean exterior chain length to that of the mean interior chain length is proportionally larger (see Table 2).

Further work is now in progress, with a view to determining the degree of multiple-branching in TFP. It seems probable that 'amylopectins' and 'glycogens' are more correctly differentiated by this property, rather than by chain length (cf. Hirst & Manners, 1954); a comparison of the degrees of multiple-branching in TFP, *T. pyriformis* glycogen and the sheep protozoal polysaccharide will therefore be of considerable interest.

SUMMARY

1. Glucose-containing polysaccharides have been isolated from the parasitic flagellates *Trichomonas foetus* and *T. gallinae*.

2. The purified polysaccharides resemble, but are not identical with, animal glycogens; they have $[\alpha]_D^{18}$ 197–199°, stain yellow-brown with dilute iodine, are degraded by salivary α-amylase and have molecular weights of *ca.* 3×10^6.

3. The 'glycogens' from *T. foetus* and *T. gallinae* have unit-chain lengths of 15 and 9 glucose residues respectively, and β-amylolysis limits of 60 and 51%.

4. The relationship between these 'glycogens' and other polysaccharides, including those from other Protozoa, are discussed.

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Methyl Mercaptan in Relation to Foetor Hepaticus

BY F. CHALLENGER
The Department of Colour Chemistry, The University, Leeds

AND J. M. WALSHE
The Medical Unit, University College Hospital Medical School, London

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The presence of a characteristic odour (foetor hepaticus) in the breath of patients with severe liver disease is a well-recognized finding. This has been described variously as 'a musty smell' (Schiff, 1946), 'the smell of a freshly opened corpse' (Himsworth, 1947) and 'a mixture of rotten eggs and garlic' (Davidson, 1949). Reviewing the literature Lichtman (1949) referred to the smell as 'amin breath' but added that there was no evidence as to the chemical nature of the compound responsible. Davidson suggested that it was a mercaptan derived from cystine or methionine, but showed that its presence was not dependent upon methionine therapy. What is probably less well recognized is that the same or a very similar odour is sometimes present in the urine of patients manifesting foetor hepaticus (Lichtman, 1949). When present in the urine the smell may be so strong as to be highly offensive. Recently a case of massive hepatic necrosis has been studied in University College Hospital, London, W.C. 1, in which the smell was very marked in the urine and opportunity was taken to isolate and identify the compound producing the odour.

Case report

E.N. a housewife aged 45 years was admitted to University College Hospital jaundiced and in light coma. The following history was obtained from her relatives and from her physician. The illness started in December 1953 with diarrhoea, anorexia and nausea; a few days later she was noticed to be lightly jaundiced. However, she did not take to her bed and after a few days her jaundice faded and she felt better. In mid-January 1954 she again felt unwell and jaundice returned together with severe vomiting. Despite rest in bed her condition did not improve and she was admitted to another hospital on 4 February 1954. There she was treated with aureomycin but her condition continued to deteriorate and after a few days she became delirious; at the same time it was observed that the size of her liver was decreasing and that she had developed a strong foetor hepaticus and neurological signs characteristic of acute hepatic failure. She was therefore transferred to University College Hospital for further treatment. This consisted of continuous infusion of 25% glucose into the superior vena cava together with careful control of the fluid and electrolyte balance, intravenous sodium glutamate and prophylactic penicillin. Paper-chromatographic examination of the plasma repeatedly showed a moderate increase in the concentration of the commonly found amino acids, but a very marked increase of methionine, as has been observed

previously in cases of severe hepatitis (Walshe, 1953). Death occurred after the patient had been in coma for 10 days. Post-mortem examination (Dr R. A. B. Drury) showed that the liver weighed only 840 g. and that this reduction in size was due to almost complete destruction of the parenchymal cells.

Whilst the patient was in University College Hospital all urine was collected over thymol and stored at 4°. It was observed that an odour very similar to the characteristic foetor hepaticus in the breath was present in the urine as soon as it was passed. It seemed probable that this odourous substance in the urine was either an alkyl mercaptan $C_nH_{2n+1}.SH$, a dialkyl sulphide $C_nH_{2n+1}.S.C_nH_{2n+1}$ or a dialkyl disulphide $C_nH_{2n+1}.S.S.C_nH_{2n+1}$ or a mixture of all three. It was decided to employ the method developed by Challenger & Rawlings (1937) and Blackburn & Challenger (1938) for identification.

EXPERIMENTAL

Examination of the urine. A slow stream of nitrogen was passed through approx. 5–6 l. of the urine and then successively through tubes containing dilute H_2SO_4 (to remove NH_3) and then through (*a*) aqueous $Hg(CN)_2$ and (*b*) aqueous $HgCl_2$. The volume of liquid in the tubes was about 5 ml. in each. A deposit formed in (*a*) which had a very faint yellow tint, gave an odour of methyl mercaptan (CH_3SH) on acidification and melted unsharply and decomposed from 150 to 180°. It was heated for some time with water, to remove $Hg(CN)_2$ from small quantities of the double compound $(CH_3S)_2Hg$, $Hg(CN)_2$, filtered and recrystallized from much hot ethanol, when it separated in shining leaflets closely resembling in appearance $Hg(SCH_3)_2$. These melted and decomposed at 170–174° and at 168° in admixture with an authentic sample of m.p. 167–168° (decomposed). $Hg(SCH_3)_2$ melts and decomposes at 174–175°. The authentic sample evidently contained traces of $Hg(CN)_2$, hence the lower m.p.

Similar m.p.'s and mixed m.p.'s were obtained with the $Hg(CN)_2$ ppt. which had been heated with water, but not recrystallized. When the m.p. tube containing the $Hg(SCH_3)_2$ from the urine was removed from the apparatus, the characteristic odour of $(CH_3)_2S$ was readily detected. This arises from the thermal decomposition:

$$Hg(SCH_3)_2 = HgS + (CH_3)_2S.$$

Its formation affords further evidence of the presence of CH_3SH in the urine. The deposit in tube (*b*) in the original experiment was much smaller in amount than that in (*a*). It gave a scarcely perceptible odour with NaOH, indicating the absence of the double compound $2(CH_3)_2S, 3HgCl_2$ and hence of $(CH_3)_2S$ from the urine (see below and Challenger & Rawlings, 1937). With dilute acid the odour of CH_3SH was readily detected. The solid sintered from 150° and decomposed at 180° without completely melting, a behaviour consistent with that of a mixture of CH_3SHgCl, $HgCl_2$ and CH_3SHgCl. The deposit in the $HgCl_2$ arose either from traces of CH_3SH, which escaped precipitation in the cyanide or, more likely, by fission of $CH_3.S.S.CH_3$ produced by atmospheric oxidation of the CH_3SH in the urine. Disulphides pass unchanged through $Hg(CN)_2$.

DISCUSSION

The high concentration of methionine observed in the blood of the patient suggests that the methyl mercaptan may have arisen from this amino acid by hydrolytic or reductive fission of the bond between the CH_3S— group and the adjoining carbon atom, giving rise to homoserine or α-aminobutyuric acid respectively as the second product. Such a fission has been observed with methionine and *S*-methylcysteine giving methyl mercaptan, both in cultures of the mould *Scopulariopsis brevicaulis* (Challenger & Charlton, 1947) and with the enzyme thionase occurring in liver tissue (Binkley, 1950).

It is well known that, in addition to acting as a source of cysteine and of other amino acids, methionine can supply a methyl group for the biological synthesis of choline, creatine, carnosine, adrenaline and *N*-methylnicotinamide. The methionine is then re-formed and is maintained at a fairly constant level. Many of these transmethylations presumably take place in the liver and in cases of severe damage to that organ, methyl transfer is possibly inhibited, with the result that methionine may accumulate; such an accumulation has actually been observed in the case of this and other patients with severe parenchymal liver disease (Walshe, 1953). The organism may then employ another mechanism, namely fission of the other carbon–sulphur link with elimination of methyl mercaptan, in order to deal with the excess of methionine. Evidence for this fission was obtained by Dent (1947), who found that, in normal subjects and especially in a case of the Fanconi syndrome, increased excretion of α-aminobutyric acid followed the administration of methionine 'as if some direct desulphurization had taken place'. The fate of the CH_3S— group of the methionine was not investigated.

The oxidation of the methyl group of methionine labelled with ^{14}C giving $^{14}CO_2$ has been studied by du Vigneaud, Mackenzie and their colleagues (for review see du Vigneaud, 1952) in intact rats and with liver and kidney-cortex slices. Mercaptan production may, of course, be preceded by oxidative deamination of methionine and formation of the corresponding α-keto acid, which has been shown by Challenger & Liu (1950) to yield methyl mercaptan and, by methylation, dimethyl sulphide in cultures of *S. brevicaulis*. Amination of the keto acid to methionine followed by fission, was, however, not excluded.

The absence of dimethyl sulphide in the urine is noteworthy and may be due to impairment of the methylation processes of the liver. In man, methyl mercaptan may be expected to be methylated, to some extent at least, to dimethyl sulphide. This sulphide is almost certainly exhaled on administration to man of thiourea, a type of mercaptan

(Challenger & Liu, 1950; Astwood, 1943*a*, *b*; Himsworth, 1943), and also on injection into dogs (Pohl, 1904). Man also converts sodium tellurite Na_2TeO_3 into dimethyl telluride, $(CH_3)_2Te$ (Bird & Challenger, 1939). The odour of the breath of the patient E.N. was less unpleasant than that of the urine. This may have been due to the oxidation of the methyl mercaptan to dimethyl disulphide, $CH_3.S.S.CH_3$, which has a less obnoxious odour. The breath was not examined chemically, so the presence of some dimethyl sulphide, $CH_3.S.CH_3$, is not excluded.

The accumulation of methionine in the blood may possibly have aggravated the toxic symptoms in the case of this patient E.N.; we know of no work on the pharmacological properties of methyl mercaptan. However, Roberts & Eckstein (1944) state that dimethyl disulphide is toxic to rats, the fatal intraperitoneal dose ranging from 90 to 100 mg./kg. body weight. Dimethyl disulphide appears to yield methyl mercaptan under these conditions and, in common with

methyl*iso*thiourea, $HN{=}C(SCH_3).NH_2$

and methylxanthate, $S{=}C(SCH_3).OC_2H_5$,

to exert a lipotropic action in rats, which is also exhibited by dimethyl sulphide (Roberts & Eckstein, 1944). Methyl mercaptan is readily produced from dimethyl disulphide by reduction and from the other two substances by hydrolysis. Some observations indicate a toxic action of methionine, when present in the diet in excess. It has been shown by Barclay, Kenney & Cooke (1945) that large doses of methionine produce ketosis in man, acetone and β-hydroxybutyric acid being excreted. Raised blood keto acid levels have been reported in patients with severe liver damage by Seligson, McCormick & Sborov (1952) and in patients in hepatic coma by Amatuzio & Nesbitt (1950). Methionine may also produce acidosis as the sulphur is metabolized principally to inorganic sulphates. Furthermore, Watson (1949) has described a patient with chronic liver disease who lapsed into coma on each occasion that methionine was given. Earle, Smull & Victor (1942) found that when methionine was fed to rats as 6·4–12 % of the McCollum stock diet, or of a low-protein and low-fat diet, severe atrophy of the liver cells, but no cirrhosis, was produced. All the rats lost weight and considerable mortality occurred in about 1 week. Roth & Allison (1950) showed that rats on a diet containing 4·8 % of methionine and 0·1 % of choline chloride lost weight, mainly owing to loss of fat. With 7 % methionine, loss of weight and excessive destruction of body tissue occurred. These authors refer to the still obscure relation between methionine and fat metabolism and cite the work of Barclay *et al.* (1945) already mentioned. An observation by Challenger, Lisle & Dransfield (1954) may have some relevance to this discussion. Cultures of *S. brevicaulis* and of *Aspergillus niger* convert sodium selenate Na_2SeO_4 into volatile dimethyl selenide, $(CH_3)_2Se$. When

$^{14}CH_3.S.CH_2.CH_2.CH.(NH_2).COOH$

was added as a methyl donor to the cultures, the yield of dimethyl selenide was considerably reduced, although 90 % of its methyl groups were found to be labelled. Presumably any substantial increase over the amount of endogenous methionine which the mould normally has at its disposal diminishes its capacity for methylation.

SUMMARY

1. A case of massive hepatic necrosis exhibited foetor hepaticus in the breath and a similar odour in the urine.

2. Passage of nitrogen through the urine into mercuric cyanide yielded mercury dimethyl mercaptide $Hg(SCH_3)_2$.

3. The probable source of the methyl mercaptan is the CH_3S— group of methionine, the normal demethylating processes being inhibited by liver damage.

4. Both methyl mercaptan and dimethyl disulphide may be exhaled in the breath.

5. Analogous reactions in certain micro-organisms, and with the enzyme thionase, and the toxicity of methionine to man and animals are considered.

Note added in proof: Sherlock *et al.* (1954) have also commented on the toxicity of methionine in cases of liver disease. It is to be inferred that they attribute this effect to ammonia formation.

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A Glucuronic-decomposing Enzyme from Rumen Micro-organisms

3. MODE OF ACTION, SPECIFICITY AND INHIBITION BY ACIDIC SUGAR DERIVATIVES*

By C. A. MARSH
Rowett Research Institute, Bucksburn, Aberdeenshire

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Other papers in this series (Karunairatnam & Levvy, 1951; Marsh, 1954) have described the discovery of a glucuronide-decomposing enzyme in micro-organisms normally present in the sheep rumen, and the partial purification of the enzyme and the kinetics of its action upon some biosynthetic glucosiduronic acids. No attempts have hitherto been made to demonstrate the hydrolytic action of the so-called β-glucuronidases of *Escherichia coli* and other micro-organisms, and while in the previous work on the rumen enzyme there was no evidence of any alternative mode of action, classification of it has been delayed until the sugar residue after enzyme action could be identified with certainty. The action upon simple α- and β-glycosides and glycosiduronic acids of the three common hexose series was examined. For this specificity study the general method, adopted previously for the study of tissue β-glucuronidase (Levvy & Marsh, 1952), whereby possible alternative substrates were tested as competitive inhibitors for the enzymic decomposition of a known standard substrate, was used throughout.

Inhibition of the glucuronide-decomposing rumen enzyme by D-glucosaccharo-1→4-lactone was noted by Levvy (1952); the negligible lactonization at the nearly neutral pH of optimum activity of the enzyme explained the negative results previously obtained by Karunairatnam & Levvy (1951) with saccharate solutions. The inhibitory effects of this and other hexaric acids and derivatives, and of the common hexuronic acids, were investigated.

The results presented in this paper show that the enzyme isolated from the sheep rumen has the same *in vitro* action and specificity as tissue β-glucuronidase, and may therefore be classified as a true β-glucuronidase.

* Part 2, Marsh (1954).

MATERIALS AND METHODS

Materials. The following compounds were synthesized: phenyl α-D-glucopyranoside (Fischer & v. Mechel, 1916), phenyl β-D-glucopyranoside (Fischer & Armstrong, 1901), (−)-menthyl α-D-glucopyranoside (Fischer & Bergmann, 1917), (−)-menthyl β-D-glucopyranoside (Treibs & Franke, 1950), methyl α-D-glucopyranoside (Helferich & Schäfer, 1948), methyl α- and β-D-galactopyranosides (Dale & Hudson, 1930), methyl α-D-mannopyranoside (Fischer, 1895), methyl β-D-mannopyranoside *iso*propanol solvate (Isbell & Frush, 1940).

From the above glycosides the following glycosiduronic acids were prepared by catalytic oxidation (Marsh, 1952): (−)-menthyl α-D-glucosiduronic acid, methyl α- and β-D-galactosiduronic acids, methyl α- and β-D-mannosiduronic acids; 3-*O*-methyl D-glucuronic acid was similarly prepared from 1:2-*O*-*iso*propylidene 3-*O*-methyl D-glucose (Vischer & Reichstein, 1944). Hydrolysis of methyl α-D-mannosiduronic acid by the method of Ault, Haworth & Hirst (1935) yielded D-mannurone, which was converted into mannuronate by treatment with dil. NaOH.

Methyl α-D-xyloside was donated by Professor E. L. Hirst to Dr G. A. Levvy, and the di-*O*-*iso*propylidene derivative of 2-keto L-gulonic acid by Dr F. Bergel; to remove the acetone groups of the latter compound, a 10% (w/v) aqueous soln. was boiled for 30 min. (Reichstein & Grüssner, 1934). The following compounds were presented by Professor F. Smith to Dr G. A. Levvy: D-glucosaccharo-1→4- and 6→3-monolactones and 1→5·6→3-dilactone (Smith, 1944), D-mannosaccharo-1→4·6→3-dilactone (Haworth, Heslop, Salt & Smith, 1944), methyl β-D-glucofuranosidurono-6→3-lactone (Owen, Peat & Jones, 1941) and methyl 4-*O*-methyl D-glucosiduronic acid amide (mixture of α- and β-forms) (Smith, 1951). By treating the corresponding lactones with dil. NaOH, solutions of D-mannosaccharate and methyl β-D-glucofuranosiduronate were prepared; the latter compound unlike phenolic glucofuranosiduronate solutions (see Tsou & Seligman, 1952), is stable at neutral pH (Owen *et al.* 1941). Methyl 4-*O*-methyl D-glucosiduronate was prepared by heating an aqueous solution of the amide with dil. NaOH (Smith, 1951), and the glycosidic linkage

was completely hydrolysed by subsequent boiling with N-HCl for 3 hr.; on neutralizing the hydrolysate with dil. NaOH the product behaved as a lactone rather than as a free acid, and it was assumed that 4-methyl D-glucurone was formed in the initial acid hydrolysis.

The following biosynthetic ester type glucosiduronic acids were presented by Professor R. T. Williams to Dr G. A. Levvy; veratroyl glucuronide (Sammons & Williams, 1946), and the potassium salts of diethyl acetyl and α-ethylhexanoyl glucuronide (Kamil, Smith & Williams, 1953).

Oxycellulose (polyanhydroglucuronic acid) (Yackel & Kenyon, 1942) was donated by Imperial Chemical Industries (Explosives) Ltd. This product had a stated carboxyl content of 22% (w/w) (theoretical COOH for polyanhydroglucuronic acid, 26%). It was brought into solution by the careful addition of dil. NaOH so that the pH did not exceed 7; the titration to neutrality gave a COOH value of 19% (w/w). D-Glucurone was donated by Corn Products Refining Co. Ltd. and was converted into glucuronate by treatment with dil. NaOH.

The following products were obtained commercially: α-D-galacturonic acid (Roche Products Ltd.), salicin (Hopkin and Williams Ltd.); L-ascorbic acid (L. Light and Co. Ltd.); potassium hydrogen saccharate, mucic acid, and D-glucono-1→5-lactone (British Drug Houses Ltd.). This last compound, m.p. 151–153° after recrystallization from ethanol, was converted into gluconate by treatment with dil. NaOH.

In general, solutions of alternative substrates or inhibitors were adjusted to pH 6·1 before addition to the assay solution, but this was omitted when testing solutions of neutral lactones. The preparation of free acids from the lactones by treatment with dil. NaOH was controlled in each case by the glass electrode, small quantities of alkali being added until equilibrium at pH 9 was reached, followed by readjustment to pH 6·1. In experiments designed to compare the effects of different treatments of the alternative substrate or inhibitor solutions, all solutions were tested simultaneously on a single enzyme preparation. All melting points are corrected.

Enzyme preparation and measurement of enzyme activity. The source of the enzyme and its preparation have already been fully described (Marsh, 1954). For experiments using possible alternative substrates or inhibitors, enzyme *C* preparations (Marsh, 1954) were employed, i.e. after two stages of $(NH_4)_2SO_4$ fractionation, followed by dialysis for 48 hr. The specific activity was then about 1000 enzyme activity units/mg. protein N, an enzyme activity unit being defined as that which liberated 1 μg. phenolphthalein/hr. at pH 6·1 from 0·0005 M phenolphthalein glucuronide under the conditions described by Karunairatnam & Levvy (1951). The liberation of phenolphthalein from 0·0005 M phenolphthalein glucuronide in 1 hr. at 38° in the presence and absence of alternative substrates or inhibitors was measured using as incubation mixture 1·0 ml. 1·5 N-H_3PO_4–NaOH buffer pH 6·1, 0·5 ml. 0·004 M phenolphthalein glucuronide pH 6·1, 2·0 ml. aqueous soln. of competing substrate or inhibitor and 0·5 ml. enzyme soln. Appropriate controls were set up using water in place of the substrate, inhibitor or enzyme. In experiments using oxycellulose or ascorbic acid, which gave a background colour after development at an alkaline pH, further controls, omitting both substrate and enzyme, were included. The assays and controls were performed in duplicate, with colour development by addition of glycine–Na_2CO_3 buffer, pH 10·7, as described by Karunairatnam & Levvy (1951).

For investigations of the products after rumen enzyme action, enzyme *C* preparations were again fractionally precipitated between the limits of 42–60% $(NH_4)_2SO_4$ saturation, followed by dialysis of the concentrated enzyme solution for 48 hr. at 15°.

Mouse-liver β-glucuronidase was prepared according to the procedure of Levvy & Marsh (1952) for use in the reducing sugar method.

EXPERIMENTAL AND RESULTS

Comparison of free phenolphthalein and glucuronic acid in enzyme digests of phenolphthalein glucuronide

A series of experiments was made to determine the relative proportions of phenolphthalein and reducing material formed in incubates of the enzyme with phenolphthalein glucuronide, a substrate which has a much higher affinity for the enzyme than others examined (Marsh, 1954).

Trial experiments showed that glucuronic acid could not be estimated by cerimetric methods in the presence of phenolphthalein or phenolphthalein glucuronide; the former interfered with the end point of the cerimetric titration (see Levvy, 1946), and phenolphthalein glucuronide gave high and variable reducing blanks. Mixtures containing up to 300 μg. phenolphthalein and 700 μg. phenolphthalein glucuronide were acidified with 5% (w/v) trichloroacetic acid (TCA), followed by continuous extraction in a micro extractor (Levvy, 1948) with ethyl acetate (A.R.) previously washed with water. After 30 min. all traces of free or conjugated phenolphthalein were extracted; all subsequent extractions with ethyl acetate were carried out for 1 hr., at the end of which the pH of the acid solution had risen to about 5·0, indicating the simultaneous removal of TCA. When standard quantities of glucuronic acid were included, cerimetric determination after ethyl acetate extraction indicated a negligible fall in reducing value.

To test the efficiency of the method, mouse-liver β-glucuronidase was initially used for the enzymic hydrolysis, using as incubation mixture 2·0 ml. 0·1 N acetic acid–NaOH buffer pH 5·2, 1·0 ml. 0·004 M phenolphthalein glucuronide pH 5·2 and 1·0 ml. enzyme soln. After incubation for 1 hr. at 38°, the solutions were rapidly cooled to 15° and 0·25 ml. withdrawn and added to 3·75 ml. 0·1 N acetic acid–NaOH buffer, pH 5·2, followed by 4 ml. glycine–Na_2CO_3 buffer, pH 10·7. After centrifuging at 1500 *g* for 15 min., free phenolphthalein was estimated. For the determination of free glucuronic acid, 2·0 ml. of the remaining incubate mixture were withdrawn and 2·0 ml. 10% (w/v) TCA added. After centrifuging at 1500 *g* for 15 min., 2·5 ml. of the supernatant were withdrawn and extracted continuously with ethyl acetate to remove phenolphthalein and unchanged substrate. Traces of ethyl acetate in the aqueous layer were removed by a current of air over the warmed solution, and the volume was made up to 5 ml.; 1·0 ml. samples were then taken for the measurement of reducing value by the procedure of Levvy (1946). The results of a typical experiment (Table 1) show that the amounts of liberated phenolphthalein and glucuronic acid were equivalent.

This experiment was then repeated using preparations of the rumen enzyme. The final substrate concentration was

Table 1. *Estimations of free phenolphthalein and reducing uronic acid (calculated as glucuronic acid) in incubates of phenolphthalein glucuronide with mouse-liver β-glucuronidase and rumen enzyme*

Enzyme	Substrate concentration (M)	Period incubation at 38°	Total liberated phenolphthalein (μg.)	Total liberated glucuronic acid: Reducing method (μg.)	Total liberated glucuronic acid: Naphthoresorcinol method (μg.)	Theoretical free glucuronic acid (μg.)
Mouse-liver β-glucuronidase	0·004	1 hr. at pH 5·2	1020	626	—	622
Rumen enzyme	0·002	24 hr. at pH 6·5	1840	1102	1137	1123

raised to 0·002 M, and 0·1 N citric acid–NaOH buffer, pH 6·5, was substituted for the acetate buffer used above. Employing the procedure described above, there was an apparent loss of about 60% free glucuronic acid, assuming this to be the liberated reducing material. It has previously been shown that free glucuronic acid present in rumen enzyme preparations is precipitated together with protein prior to the estimation of reducing value (Marsh, 1954). The following modifications were finally made: after 24 hr. incubation at 38°, followed by addition of TCA, 2·5 ml. of the mixture was immediately removed and extracted with ethyl acetate without prior removal of precipitated protein. Further material, probably mucopolysaccharide, was observed to coagulate at the water/ethyl acetate interface. After removal of ethyl acetate, the aqueous suspension was allowed to stand for 12 hr. at 15°, then diluted to 5 ml. and centrifuged at 1500 *g* for 15 min. Reducing value estimations on the supernatant were carried out as before, together with estimation of non-extractable uronic acid by the Tollens reaction with naphthoresorcinol, using the procedure of Hanson, Mills & Williams (1944). The uronic acid was calculated as D-glucuronic acid from a standard curve. As shown in Table 1, similar values for liberated glucuronic acid were given by the reducing method and by the naphthoresorcinol reaction, and these were also equivalent to the amount of aglycone liberated.

Identification of free D-*glucuronic acid from enzyme digests of phenolphthalein glucuronide*

By paper chromatography. After incubating the rumen enzyme solution (1·0 ml.) with 0·005 M phenolphthalein glucuronide (1·0 ml.), pH 6·5, for 24 hr. at 38°, followed by dialysis into water for 24 hr. at 0°, the dialysate was found to contain 450 μg. free glucuronic acid, estimated cerimetrically. The dialysate was evaporated to dryness *in vacuo*, and extracted with 0·2 ml. water. Chromatographic separation was then carried out on paper in an amyl alcohol–pyridine–water mixture (7:7:6, by vol.) (Jeanes, Wise & Dimler, 1951), together with authentic samples of D-glucurone, D-glucuronic acid, D-galacturonic acid and D-xylose. There was no interference in the separation of the reducing sugars in the presence of phenolphthalein or its glucuronide nor from dialysable products in the enzyme preparation. After development, the papers were sprayed with *o*-phenetidine and benzidine to demonstrate the presence of uronic acids and xylose respectively. Only one spot from the dialysed enzyme digest was observed, and this corresponded to D-glucuronic acid.

By isolation of the dibenziminazolyl derivative of D-*glucosaccharic acid.* An authentic specimen of the di-(benziminazol-2-yl) derivative of D-glucosaccharic acid was prepared from 180 mg. D-glucurone by oxidation and coupling with *o*-phenylenediamine (British Drug Houses Ltd.) according to the procedure of Lohmar, Dimler, Moore & Link (1942) as modified by Levvy (1948). This yielded 110 mg. (30% of theory) of the dibenziminazolyl derivative, m.p. 240° (decomp.). The dihydrochloride and dipicrate were prepared from this as described by Levvy (1948). The dihydrochloride had m.p. 267–268° (decomp.), $[\alpha]_D^{21} + 52{\cdot}0 \pm 0{\cdot}5°$ in water (*c*, 2); m.p. of dipicrate 212° (decomp.) with change of form at 144°.

For the enzymic hydrolysis, 25 ml. of the rumen enzyme preparation, containing 160 enzyme units/ml., were incubated with 25 ml. 0·03 M phenolphthalein glucuronide, pH 6·4, for 60 hr. at 38°; the pH was checked at intervals of 12 hr. The solution was then dialysed into 500 ml. water at 0° for 48 hr. The dialysate was acidified to pH 1·3 with 5 N-H_2SO_4, and extracted 10 times with ethyl acetate (total 1 l.). After testing for the absence of free or conjugated phenolphthalein, the aqueous residue was concentrated *in vacuo* to 100 ml. and shaken with $BaCO_3$ to remove sulphate. The aqueous soln. was filtered and estimated by the method of Hanson *et al.* (1944) to contain 75 mg. glucuronic acid (including volume of solution in dialysis sac); this would correspond to 52% hydrolysis, but this value is low due to retention of free glucuronic acid by the undialysable residue.

This free uronic acid solution was then oxidized and condensed with *o*-phenylenediamine as described above. Before condensation, a small sample was boiled for 1 hr. at pH 3 and tested as an inhibitor for mouse-liver β-glucuronidase as described by Levvy (1952), from which it was calculated that about 21 mg. total D-glucosaccharic acid was present (29% of theory on oxidation). This yielded 28 mg. of the dibenziminazole derivative (23% of theory from the glucuronic acid content), m.p. 238–240° (decomp.), from which were prepared the dihydrochloride m.p. 266° (decomp.) and the dipicrate m.p. 211–213° (decomp.) with change of form at 142°. None of these derivatives, when mixed with the corresponding authentic specimens, produced any depression of the melting points.

Effects of possible alternative substrates upon the enzymic hydrolysis of phenolphthalein glucuronide

Possible alternative substrates which had no appreciable effect upon the liberation of phenolphthalein from 0·0005 M phenolphthalein glucuronide by the rumen enzyme are shown in Table 2. The remaining substances, which caused a noticeable change in the liberation of phenolphthalein when present in the incubation mixture, are shown in Table 3.

Table 2. *Possible alternative substrates having negligible effect on rate of liberation of phenolphthalein from* 0·0005 M *phenolphthalein glucuronide at* pH 6·1 *by rumen enzyme*

Compound	Final concentration (M)	Change in rate of phenolphthalein liberation (% control)
Methyl α-D-glucopyranoside	0·01	0
Phenyl α-D-glucopyranoside	0·01	0
Phenyl β-D-glucopyranoside	0·01	0
(−)-Menthyl α-D-glucopyranoside	0·001	0
Methyl α-D-galactopyranoside	0·01	0
Methyl β-D-galactopyranoside	0·01	0
Methyl α-D-mannopyranoside	0·01	0
Methyl β-D-mannopyranoside	0·01	−2
Salicin	0·01	−2
Cellobiose	0·01	0
Methyl β-D-xylopyranoside	0·01	0
Methyl α-D-galactosiduronic acid	0·01	0
Methyl β-D-galactosiduronic acid	0·01	0
Methyl α-D-mannosiduronic acid	0·01	0
Methyl β-D-mannosiduronic acid	0·01	−2
Methyl 4-*O*-methyl D-glucosiduronic acid*	0·002	0
Methyl β-D-glucofuranosiduronic acid	0·001	−2

* Mixture of α- and β- anomers.

Table 3. *Possible alternative substrates affecting the rate of liberation of phenolphthalein from phenolphthalein glucuronide at* pH 6·1 *by rumen enzyme*

Compound	Final concentration (M)	Standard substrate concentration (M)	Change in rate of phenolphthalein liberation (% control)
(−)-Menthyl β-D-glucopyranoside	0·001	0·0005	+4
(−)-Menthyl α-D-glucosiduronic acid	0·01	0·0005	+8
Oxycellulose (freshly prepared)	0·01*	0·0005	−15
Veratroyl glucosiduronic acid	0·0005	0·0002	−29
Diethylacetyl glucosiduronic acid	0·004	0·0002	−21
α-Ethylhexanoyl glucosiduronic acid	0·001	0·0002	−22

* Calculated as glucurone.

Table 4. *Inhibitions of oxycellulose solutions on the liberation of phenolphthalein from* 0·0005 M *phenolphthalein glucuronide by rumen enzyme*

For preparation of solutions see text. Concentrations are calculated as glucurone.

Preparation	Concentration (M)	Inhibition of rate of phenolphthalein liberated (%)
Freshly prepared soln.	0·01	15
Soln. kept 48 hr.	0·01	11
Residue after complete dialysis	0·008*	6–9
Dialysate	0·001*	7

* Calculated from results obtained by dialysing a soln. at pH 7 into water for 48 hr. at 15°.

Definite inhibitory effects were produced only by oxycellulose and the ester glucuronides of veratric, diethylacetic and α-ethylhexanoic acids. To conserve material, the concentrations of the last three compounds were considerably reduced, and the standard substrate concentration was accordingly decreased to 0·0002 M.

Direct measurements of the liberation of reducing material after incubating oxycellulose with the enzyme could not be made, since this material is very unstable at an alkaline pH. Its inhibitory power after dialysis was investigated, however. A solution of oxycellulose (0·02 M, calculated as D-glucurone) in 0·3 N-H_3PO_4–NaOH buffer, pH 6·1, was dialysed for 48 hr. at 15° into an equal volume of the same buffer. A similar solution was dialysed for the same period into several changes of fresh buffer to remove all dialysable material; approximately 20 % was lost in this time. The inhibitory powers of the first dialysate, the residue from complete dialysis, and a non-dialysed solution allowed to stand at room temperature were then compared (Table 4). The inhibitory power decreased after dialysis, and the inhibition caused by the dialysate at the lower concentration was as high as that of the undialysable residue.

Effects of possible inhibitors on the enzymic hydrolysis of phenolphthalein glucuronide

Results of preliminary tests for the inhibition of the liberation of phenolphthalein from 0·0005 M phenolphthalein glucuronide by the rumen enzyme, in the presence of acidic sugar derivatives, are shown in Table 5.

Table 5. *Effects of acidic sugar derivatives and their lactones on rate of liberation of phenolphthalein from* 0·0005 M *phenolphthalein glucuronide by rumen enzyme*

Compound	Final concentration (M)	Inhibition of rate of phenolphthalein liberation (%)
D-Gluconate	0·01	2
D-Gluconate (after 1 hr. at 100° and pH 2)	0·01	4
D-Glucono-1→5-lactone	0·01	2
D-Glucuronate	0·01	21
D-Glucuronate	0·002	11
3-*O*-Methyl D-glucuronate	0·002	5
4-*O*-Methyl D-glucuronate	0·002	3
D-Glucurone	0·01	3
4-*O*-Methyl D-glucurone*	0·002	0
D-Galacturonate	0·01	0
D-Mannuronate	0·01	2
D-Mannurone	0·01	2
D-Glucosaccharate	0·01	6
D-Glucosaccharate (after 6 hr. at 20° and pH 3)	0·01	12
D-Glucosaccharate (after 1 hr. at 100° and pH 3)	0·001	49
D-Glucosaccharo-1→4-lactone	0·00025	49
D-Glucosaccharo-6→3-lactone	0·01	18
D-Glucosaccharo-1→5-6→3-dilactone	0·001	6
Mucate (D-galactosaccharate)	0·005	−7
Mucate (after 2 hr. at 100° and pH 2)	0·005	0
D-Mannosaccharate	0·001	2
D-Mannosaccharo-1→4-6→3-dilactone	0·001	3
L-Ascorbate	0·01	73†
L-Ascorbate	0·001	7
2-Keto-L-gulonate	0·002	4

* Initial product from methyl 4-*O*-methyl-D-glucosiduronic acid after acid hydrolysis.

† This value is probably high since 0·01 M ascorbic acid interferes with the phenolphthalein colour reaction.

Of particular interest were the inhibitory effects caused by the hexaric acids and their lactones. Whereas freshly prepared D-glucosaccharic acid solutions had little inhibitory power, after a period of ageing at an acid pH, and particularly on boiling for a short time, marked inhibition was caused. The inhibition by the 1→4-lactone, of D-glucosaccharic acid was relatively so powerful that the inhibitions caused by the 6→3-lactone and 1→5:6→3-dilactone could well be explicable as due to a trace (about 0·5 %) of the 1→4-lactone in these preparations. The inhibition of the rumen enzyme by D-glucosaccharo-1→4-lactone was quantitatively independent of the period of incubation at 38° and pH 6·1, indicating that the inhibitor is stable at this pH.

A solution of D-glucosaccharo-1→4-lactone had a similar inhibitory power for enzyme preparations of varying purity, and inhibited the liberation of phenolphthalein from its glucuronide by the intact micro-organisms. Table 6 shows the effect upon enzyme preparations at stages *A*, *B* and *C*, and a suspension of active micro-organisms, prepared according to the procedure of Marsh (1954). Microscopic examination of the suspension before incubation showed the organisms to be then still viable.

The kinetics of the inhibition of the enzymic hydrolysis of phenolphthalein glucuronide by 0·01 M D-glucuronic acid and 0·00025 M D-glucosaccharo-1→4-lactone at varying substrate concentrations were investigated. In both cases percentage inhibition decreased with increasing substrate concentration, showing that inhibition was competitive. Using the procedure previously adopted (Marsh, 1954) for the determination of the Michaelis constant K_s for alternative substrates for the rumen enzyme, mean values of $3{\cdot}6 \times 10^{-3}$ M for D-glucuronic acid, and $1{\cdot}9 \times 10^{-5}$ M for D-glucosaccharo-1→4-lactone, were obtained for K_i, the apparent dissociation constant of the enzyme-inhibitor complex.

DISCUSSION

Detection and assay of the rumen enzyme have hitherto always been made by colorimetric measurement of the liberated aglycone. It is clear from the

Table 6. *Inhibition by* 0·00025 M *D-glucosaccharo-1→4-lactone of liberation of phenolphthalein from* 0·0005 M *phenolphthalein glucuronide by suspensions of rumen micro-organisms and by rumen enzyme preparations*

For details of enzyme purification, see Marsh (1954).

Stage of enzyme purification	Inhibition of rate of phenolphthalein liberation (%)
Suspension of active micro-organisms	44
Enzyme *A* (crude cell-free extract)	46
Enzyme *B* (after 1st $(NH_4)_2SO_4$ fractionation)	49
Enzyme *C* (after 2nd $(NH_4)_2SO_4$ fractionation)	49

experimental evidence, however, that the enzyme has a hydrolytic *in vitro* action; it is thus a β-glucuronidase. The direct chemical isolation of glucuronic acid liberated in enzyme digests is difficult, although Miwa (1932) has isolated the potassium salt from incubates of flavone glucosiduronic acids with baicalinase, a plant enzyme which has now also been identified as a β-glucuronidase (Levvy, 1954). The oxidation method of Lohmar *et al.* (1942), whilst giving low yields, is more convenient for the identification of small quantities of glucuronic acid such as were obtainable from digests with the rumen enzyme. The equivalence of liberated reducing uronic acid, calculated as D-glucuronic acid, with the amount of free aglycone in enzyme digests is further strong evidence of a hydrolytic action.

The substrate specificity of the rumen enzyme seems to be very similar to that of animal tissue β-glucuronidase, as described by Levvy & Marsh (1952), in that the rumen enzyme is specific for the hydrolysis of β-D-glucosidpyranuronic acids. The inhibition of the enzymic hydrolysis of the standard substrate by ester glucosiduronic acids may be due to their effect as alternative substrates or as true inhibitors. The lability of these compounds in alkaline solution (Pryde & Williams, 1933) has made the direct investigation of their hydrolysis difficult, but Levvy (1954) has stated that these compounds are substrates for mouse-liver β-glucuronidase, and this is therefore probably also true for the rumen enzyme.

Oxycellulose has a slightly lower inhibitory effect than D-glucuronic acid on the rumen enzyme, but the inhibition was apparently associated mostly with the dialysable material in the preparation. Since the latter formed only 20% of the total weight, the inhibition could not be accounted for by glucuronic acid formed by complete hydrolysis. The inhibitory material in oxycellulose would thus seem to be polysaccharides of low molecular weight, and these may be substrates for the enzyme. It is therefore interesting to note that there was no experimental evidence of an action of the rumen enzyme upon methyl 4-*O*-methyl β-D-glucosiduronic acid. This suggests that if the function of this and other similar alimentary enzymes in herbivores is to break down hemicellulose material, as has been previously suggested (Marsh, Alexander & Levvy, 1952; Robinson, Smith & Williams, 1953; Billett, 1954), the site of such enzyme action is probably only at the linkage of a terminal glucuronic acid residue at the non-reducing end of the polysaccharide. This may also be true of animal tissue β-glucuronidase, on which the effects of oxycellulose and substituted glycosiduronic acids are similar to those on the rumen enzyme (Levvy & Marsh, 1952).

The slight activation of rumen β-glucuronidase activity by (−)-menthyl α-D-glucosiduronic acid and (−)-menthyl β-D-glucoside resembles the effects of (−)-menthol derivatives on the tissue enzyme (Levvy & Marsh, 1952), but the rumen enzyme differs from the latter in that no activation was observed in the presence of the alternative substrate (−)-menthyl β-D-glucosiduronic acid (Marsh, 1954). A saturated solution of (−)-menthol had no appreciable effect.

Of the compounds examined as possible true inhibitors of rumen β-glucuronidase, D-glucosaccharo-1 → 4-lactone was by far the most powerful, causing 50% inhibition of enzyme activity at a concentration of 0·00025 M with substrate concentration 0·0005 M. This confirms the results previously obtained by Levvy (1952). The inhibitory power was appreciably lower for the rumen enzyme than for mouse-liver β-glucuronidase, for at the same inhibitor concentration over 90% inhibition of animal tissue β-glucuronidase activity was noted with a substrate concentration of 0·00063 M, and a 0·000004 M lactone solution caused 50% inhibition under the same conditions (Levvy, 1952). The inhibition of the rumen β-glucuronidase by acidified D-glucosaccharic acid preparations was correlated with formation of the 1 → 4-lactone in these solutions as found by Levvy (1952). Since this inhibitor was found to be stable at the normal pH (5·9–6·2) of the sheep rumen, and appeared to inhibit effectively the glucosiduronic acid-decomposing ability of the whole organisms, it may prove of value in attempting to elucidate the physiological function of the enzyme.

There is a marked difference between rumen and tissue β-glucuronidases in their susceptibilities to inhibition by mucic acid preparations. The boiling of a mucic acid solution at an acid pH is said to result in rapid lactone formation (Levene & Simms, 1925), although the evidence, largely titrimetric, for this is circumstantial, for no lactones of mucic acid have been isolated. Levvy (1952) found that boiled mucic acid solutions (0·0007 M) caused strong inhibition of mouse-liver β-glucuronidase activity, whereas unboiled mucic acid (0·002 M) had little effect. In the case of rumen β-glucuronidase, boiled mucic acid at the latter concentration had no effect, whereas unboiled preparations caused slight activation of enzyme activity (Table 5). This difference was not due to changes in composition of mucate solutions at pH 5·2 and 6·1, for by experiment there was found to be equally strong inhibition of mouse-liver β-glucuronidase at pH 5·2 (acetate buffer) and pH 6·1 (phosphate buffer).

D-Glucuronic acid, a product of the enzyme action, caused inhibition of rumen β-glucuronidase, but the monomethyl derivatives had less effect, and D-glucurone and (hypothetical) 4-*O*-methyl D-glucurone were apparently inactive. In this respect

the rumen enzyme closely resembles tissue β-glucuronidase (see Karunairatnam & Levvy, 1949; Spencer & Williams, 1951; Levvy, 1952), but inhibition of the latter by D-glucuronic acid is more marked.

Quantitative measurement of the enzyme activity in the presence of high concentrations of L-ascorbic acid was difficult, but some inhibition was apparent. Becker & Friedenwald (1949) found that this compound strongly inhibited rat-liver β-glucuronidase (see also Mills, Paul & Smith, 1953). Levvy (1952) found no inhibition of mouse-liver β-glucuronidase by 0·001 M ascorbic acid, and at 0·01 M concentration Wong & Rossiter (1951) found negligible inhibition of rabbit polymorphonuclear leucocyte β-glucuronidase. No satisfactory explanation has been given for these varying results with animal tissue β-glucuronidase preparations.

In the case of D-gluconic acid, any inhibitory power for β-glucuronidase seems to be associated with an impurity. In earlier experiments it was noted that solutions of 0·01 M gluconic acid after boiling at pH 2 for 1 hr. caused up to 10 % inhibition of rumen enzyme activity (substrate concentration 0·0005 M), but the inhibitory effect became negligible after several recrystallizations of the D-glucono-1 → 5-lactone. Some authors, e.g. Karunairatnam & Levvy (1949), and Mills *et al.* (1953) have described slight inhibition by gluconate of tissue β-glucuronidase acting on phenyl glucuronide, whereas Wong & Rossiter (1951) and Levvy (1952) found no effect using phenolphthalein glucuronide as the substrate. These differing results might partly be due to the higher affinity of phenolphthalein glucuronide for the enzyme compared with phenyl glucuronide, but a positive effect with gluconate or gluconolactone might well be due to traces of saccharic acid formed during their manufacture.

From this survey of the inhibitory effects of acidic sugar derivatives and their lactones upon rumen β-glucuronidase, it seems that the essential requirements for an effective specific inhibitor, as for the β-glucuronidase from animal tissues, are that the configuration of the secondary hydroxyl groups of the D-glucose molecule must be preserved and unsubstituted in a cyclic structure containing a carboxylic acid grouping at the $C_{(5)}$ position, i.e. there must be a close resemblance to a β-D-glucopyranuronide structure.

SUMMARY

1. The glucuronide-decomposing enzyme in the sheep rumen was found to be a true β-glucuronidase. Phenolphthalein and glucuronic acid were liberated in equivalent amounts by enzymic action on the conjugated glucosiduronic acid.

2. Liberated glucuronic acid was identified by oxidation to D-glucosaccharic acid, from which derivatives were prepared.

3. Rumen β-glucuronidase was found to be specific for β-D-glucosidpyranuronic acids, and had no effect upon glycosides or on glycosiduronic acids of other sugars, nor upon methyl 4-*O*-methyl glucosiduronic acid.

4. Acylglucuronides and dialysable components of oxycellulose inhibited enzyme action upon phenolphthalein glucuronide. These compounds may be substrates for the enzyme.

5. D-Glucosaccharo-1 → 4-lactone was a strong competitive inhibitor of rumen β-glucuronidase, although its affinity for this enzyme is lower than for tissue β-glucuronidase.

6. Boiled mucic acid preparations had no effect upon the rumen enzyme, in which respect the latter differs from tissue β-glucuronidase.

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The *N*-Terminal Amino Acid Residues of Gelatin

3. ENZYMIC DEGRADATION

By A. COURTS
The British Gelatine and Glue Research Association, 2a Dalmeny Avenue, London, N. 7

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The specific action of proteolytic enzymes on small peptides is well known (for reviews see Neurath & Schwert, 1950; Smith, 1951). The work reported in the present paper describes the hydrolytic effect of pepsin, trypsin, chymotrypsin and papain on gelatin using the *N*-terminal residue technique to follow the extent of degradation and to examine enzyme specificity. The *N*-terminal residue technique was employed in Part 2 of this series (Courts, 1954*b*) to examine thermal degradation of gelatin catalysed by hydrogen and hydroxyl ions. The technique does not permit the bonds broken to be specified, but indicates the amino acid residues carrying the amino groups which are released during hydrolysis.

MATERIALS AND METHODS

Gelatin. An alkali-processed ox-bone gelatin of isoionic point pH 5·1 was used, being identical with the material used in Part 2 of this series. The substrate was 2·5 g. in 50 ml. water adjusted to a suitable pH value.

Trypsin. Crystallized trypsin (Armour Laboratories, Chicago, U.S.A.) (0·02 g.) was used at pH 8·5. The reaction was allowed to proceed for 2·5 hr. at 21, 35, 44, 56 and 65°. The author is most grateful to Dr Watson of the Courtauld Laboratories for a gift of this enzyme.

Pepsin. Crystallized pepsin (Armour Laboratories) (0·02 g.) was used at pH 1·87 and 37°. The reaction was carried out for 6 and 19 hr. Controls were run without enzyme to allow for thermal degradation at this pH value.

Chymotrypsin. Crystallized chymotrypsin (Armour Laboratories) (0·02 g.) was used at pH 8·0 and 37°. Reaction times were 2, 4 and 6 hr. A second series was carried out to observe the effect of enzyme concentration and of variation of pH; thus 0·002 g. of chymotrypsin was incubated at 37° for 4 hr. with solutions of substrate at pH 5·1 (isoionic point of the gelatin) and at pH 8·0 (reputed optimum for this enzyme).

Papain. Commercial papain (Norman Evans and Rais, Manchester) (0·002 g.) was used at 37°. Hydrolysis was allowed to continue for 2 and 5 days at (*a*) pH 5·05, the pH optimum of papain for partially hydrolysed protein and (*b*) pH 6·95, the pH optimum for intact protein substrates. The extent of thermal degradation under these conditions was also examined.

N-Terminal residue determinations

The enzymically degraded gelatins were adjusted to pH 8·5 and allowed to react with 1:2:4-fluorodinitrobenzene (FDNB). The preparation of dinitrophenyl (DNP) derivatives, their subsequent hydrolysis and the evaluation of *N*-terminal residues were all carried out using the methods previously described by Courts (1954*a*, *b*).

Considerations of proteinase inactivation

No attempts were made to remove or inactivate the proteinase before allowing its digest to react with FDNB. Pepsin would be automatically inactivated at pH 8·5. It is likely that chymotrypsin would be inactivated by FDNB through substitution at its tyrosyl phenolic groups (cf. Sizer, 1945). Papain may likewise be inactivated by blocking of sulphydryl groupings (Smith, 1951), although for both

these reactions there may be delay before they are complete. There is no evidence to show that the active centres of trypsin are affected by dinitrophenyl or similar substitution. Since the principal objective was to study the amino end-groups released, it would not materially affect any conclusions if some tryptic hydrolysis continued into the early stages of the FDNB reaction.

RESULTS

Papain. After digesting the gelatin with papain the *N*-terminal residues detected were qualitatively similar to those obtained in intact gelatin (Courts, 1954*a*) and in gelatin which had been thermally degraded. Quantitative measurements showed that the number of end groups had substantially increased (Table 1), and that the enzyme exhibited a marked specificity for peptide bonds involving the amino groups of alanine. Both the amount of degradation and the degree of specificity were considerably more pronounced for digestions at pH 5 than at pH 7.

Chymotrypsin. The change in end groups when chymotrypsin was used for digesting gelatin is summarized in Table 2. Observations reported in Table 1 suggest that thermal degradation under these conditions was negligible. All the *N*-terminal residues corresponded to those originally found in undegraded gelatin and were present in similar proportions but substantially increased in amount. Certain other points are illustrated by Table 2: (i) contrary to the findings with papain, chymotrypsin was more vigorous at the normal pH optimum than at pH 5·1; (ii) the effect of increasing enzyme concentration by 1000% only increased the number of peptide bonds broken by about 60%; (iii) the rate of breakdown was initially very rapid, falling off gradually after 2 hr., suggesting exhaustion of some readily attacked bonds.

Pepsin. The rate of peptic hydrolysis of gelatin was comparatively slow in the conditions used. An increase occurred in all the *N*-terminal residues found in the non-degraded material. There was an 'above average' attack on peptide bonds involving alanine groups (×8 at 6 hr.; ×12 at 19 hr.) and similarly for valine, the major constituent of others in the table (×9 at 6 and 19 hr.). There was, correspondingly, a 'below average' attack on bonds involving glycine groups. This is illustrated in Table 3.

Table 1. *Liberation of* N*-terminal residues during degradation of gelatin by papain*

For conditions, see text. End groups are expressed as moles/100000 g. gelatin. M_n is number-average molecular weight calculated from end groups.

		Thermal degradation				Enzymic degradation			
		pH 5·05		pH 6·95		pH 5·05		pH 6·95	
	Undegraded	2 days	5 days	2 days	5 days	2 days	5 days	2 days	5 days
Glycine	0·95	0·97	1·23	1·00	1·21	7·10	14·00	2·44	6·32
Serine	0·29	0·31	0·30	0·27	0·33	2·28	2·40	0·48	1·05
Threonine	0·12	0·10	0·15	0·18	0·16	2·16	4·00	0·57	1·00
Alanine	0·14	0·16	0·16	0·14	0·19	9·03	12·24	1·18	4·61
Aspartic acid	0·08	0·11	0·16	0·16	0·16	1·28	3·42	0·41	0·77
Glutamic acid	0·14	0·15	0·15	0·16	0·20	0·49	1·78	0·20	0·57
Others	0·00	0·14	0·14	0·14	0·15	1·10	1·50	0·24	1·10
Total	1·73	1·94	2·29	2·05	2·40	23·44	39·34	5·52	15·42
M_n	58000	52000	44000	50000	42000	4300	2500	17000	6400
Approx. increase in glycine						×7	×14	×2½	×6
Approx. increase in alanine						×80	×100	×10	×30

Table 2. *Degradation of gelatin by chymotrypsin*

For conditions, see text. End groups are expressed as moles/1000000 g. gelatin. M_n is number-average molecular weight calculated from end groups.

		8 mg. enzyme/g. gelatin pH 8·0			0·8 mg. enzyme/g. gelatin	
	Undegraded	2 hr.	4 hr.	6 hr.	pH 8·0 4 hr.	pH 5·1 4 hr.
Glycine	0·95	5·08	7·56	8·34	4·53	2·4
Serine	0·29	0·90	0·92	1·25	0·92	0·59
Threonine	0·12	0·52	0·62	0·71	0·43	0·29
Alanine	0·14	1·00	1·24	1·40	1·00	0·57
Aspartic acid	0·08	0·55	0·61	0·75	0·36	0·33
Glutamic acid	0·14	0·37	0·58	0·85	0·33	0·16
Others	0·00	1·00	1·50	2·20	0·90	0·57
Total	1·73	9·42	13·03	15·50	8·48	4·92
M_n	58 000	10 000	7 200	6 000	11 000	19 200

Table 3. *Degradation of gelatin with pepsin*

For conditions, see text. End groups are expressed as moles/100000 g. gelatin. M_n is number-average molecular weight calculated from end groups.

	Undegraded	Thermal degradation 6 hr.	Thermal degradation 19 hr.	Enzymic degradation 6 hr.	Enzymic degradation 19 hr.
Glycine	0·95	1·09	1·20	1·89	2·81
Serine	0·29	0·28	0·27	0·58	0·68
Threonine	0·12	0·16	0·17	0·65	0·72
Alanine	0·14	0·17	0·17	1·41	2·14
Aspartic acid	0·08	0·15	0·19	0·41	0·69
Glutamic acid	0·14	0·15	0·11	0·19	0·45
Others	0·00	0·00	0·20	1·20	2·00
Total	1·73	2·00	2·31	6·33	9·49
M_n	58 000	50 000	43 300	15 700	10 500

Table 4. *Degradation of gelatin by trypsin*

For conditions, see text. End groups are expressed as moles/100000 g. gelatin. M_n is number-average molecular weight calculated from end groups.

	21°	35°	44°	56°	65°
Glycine	27·26	36·92	40·44	28·51	22·20
Serine	0·80	0·81	1·17	1·05	1·18
Threonine	1·09	1·50	1·36	1·22	1·03
Alanine	0·71	0·46	0·85	0·82	0·83
Aspartic acid	0·89	1·07	1·25	0·74	0·62
Glutamic acid	1·36	0·43	0·80	0·51	0·80
Others	0·90	1·50	1·30	1·10	2·30
Total	33·01	42·69	47·17	33·15	28·96
M_n	3 000	2 300	2 100	3 000	3 450

Trypsin. While a small increase in all the expected *N*-terminal residues occurred during tryptic hydrolysis of gelatin, by far the greater number were derived from peptide bonds involving the amino group of glycine (see Table 4). This is prevalent throughout the temperature range examined (21–65°). The temperature optimum appears to be at about 44° for the 2·5 hr. digestion.

Artifact. It was observed during these experiments involving the mild degradation of gelatin at 37°, that the DNP-derivatives on hydrolysis gave rise to a slow moving artifact (*R*, 0·02) on a Celite column buffered at pH 4 using chloroform–ether (90:10 by vol.) as the organic phase eluent. This *R* value does not correspond to a DNP-derivative of any of the amino acids known to occur in gelatin hydrolysates, neither does it correspond to DNP-glucosamine (*R*, 0·12), DNP-chondrosamine (*R*, 0·12) or DNP-β-hydroxyglutamic acid (*R*, 0·33).

DISCUSSION

The general conclusion from results for the four enzymes used is that specificity is much less restricted than for peptide substrates, and, where it occurs, it does not correspond with the results for peptides. The classical investigations of Fruton & Bergmann (1939), using pepsin on synthetic substrates, suggested that tyrosine and phenylalanine would occur as the principal *N*-terminal residues of peptic digests. This has not been found with gelatin. In this instance the enzyme has attacked more readily bonds involving the amino groups of alanine, and valine, but in addition many other bonds are attacked. Other protein–pepsin hydrolysates examined by the Sanger technique also afford some evidence of multiple specificity. These are compared in Table 5.

The modes of attack of the four proteinases on gelatin are compared in Table 6. The results suggest that a number of types of bonds are attacked by all four enzymes. Somewhat similar conclusions were reached by Rovery, Desnuelle & Bonjour (1950) from observations on horse globin hydrolysed by pancreatic proteinases. The development of end-group methods of analysis is likely to provide further evidence of discrepancies between enzymic specificity towards small peptide units and large protein units. Thus, Gladner & Neurath (1952) have shown that the *C*-terminal residues resulting from the tryptic conversion of chymotrypsinogen into chymotrypsin are tyrosine and leucine, whereas tryptic hydrolysis of synthetic substrates has led to rupture at the carboxyl groupings of arginine and lysine (Neurath & Schwert, 1950).

It appears likely that the emphasis placed upon the importance of synthetic substrates for elucidating protein structure (Smith, 1951; Neurath & Schwert, 1950; also others) may have to be reviewed. The possibility arises that a proteinase reacts in two

Table 5. *N-Terminal residues released in proteins by pepsin*

Protein	Reference	Primary specificity	Secondary specificity
Gelatin	Present work	Alanine, valine	Glycine, serine, threonine, aspartic acid, glutamic acid
Ovalbumin	Desnuelle, Rovery & Bonjour (1950)	Complex mixture	—
Horse globin	Desnuelle *et al.* (1950)	Alanine, phenylalanine	Leucine, serine
Oxidized insulin A chain	Sanger & Thompson (1953)	Tyrosine, glutamic acid, aspartic acid	Leucine, cysteic acid
Oxidized insulin B chain	Sanger & Tuppy (1951)	Tyrosine, valine, leucine, phenylalanine	Alanine, histidine

Table 6. *N-Terminal residues released during the proteolysis of gelatin*

Proteinase	Primary specificity	Secondary specificity
Pepsin	Alanine, valine	Serine, threonine, aspartic acid, glutamic acid, glycine
Trypsin	Glycine	Serine, threonine, aspartic acid, glutamic acid, alanine
Chymotrypsin	—	Serine, threonine, aspartic acid, glutamic acid, alanine, glycine, valine
Papain	Alanine	Serine, threonine, aspartic acid, glutamic acid, glycine

distinctly different ways. First, it appears to be acting as a true catalyst, enabling aqueous hydrolysis to occur at greatly reduced temperatures. Under these circumstances it is probable that the peptide bonds, which are most sensitive to thermal hydrolysis, would be the most labile, and it may be significant that the *N*-terminal residues found in all the gelatin digests examined are the same as those released during aqueous hydrolysis at high temperatures in the presence of acid or alkali (Courts, 1954*b*). In this respect wool keratin is very similar to gelatin. Native wool shows a wide variety of *N*-terminal residues (Middlebrook, 1951) qualitatively identical with those of gelatin. Middlebrook (1951) showed that degradation of wool with hydrochloric acid provided polypeptide units involving the same end groups. Digestion of wool with papain, in the presence of urea and sodium bisulphite, again yields material with *N*-terminal residues which, except for leucine, are identical with those in the native protein, although quantitatively enhanced (Blackburn, 1950). The second mode of action appears to involve enzyme specificity. Although Desnuelle *et al.* (1950) consider that specific bond-breaking activity precedes random bond breaking, there is no evidence for this in the present work. Enzymic hydrolysis of oxidized insulin chains (Sanger & Tuppy, 1951; Sanger & Thompson, 1953) shows specificities similar in some instances to synthetic peptide substrates, together with secondary specificities. This indicates that the specificity experienced by the synthetic peptides may arise in protein digests when the polypeptide chains are small enough.

SUMMARY

1. Gelatin has been degraded by pepsin, trypsin, chymotrypsin and papain. The *N*-terminal residues of the hydrolysates were determined by Sanger's fluorodinitrobenzene technique.

2. Attacks on similar peptide bonds are shown in all four cases, although to different degrees so far as observations from the amino end can discern. Papain, for example, attacks bonds involving alanine, and, to a lesser extent, glycine, serine, threonine, aspartic acid and glutamic acid. The results are related to those of certain other workers using different proteins.

3. It is suggested that proteolysis occurs in two ways; one involving a random breaking of peptide bonds, which closely follows the course of thermal degradation, the other involving a specific bond breaking action.

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Fish Muscle Riboside Hydrolases

By H. L. A. TARR
Pacific Fisheries Experimental Station, Vancouver, Canada

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During an investigation concerning the cause and control of Maillard browning in heated flesh materials it was found that free ribose, in concentrations which occasionally exceeded 0·1 % of the wet weight, appeared when muscles of certain fishes were stored at 0° or at higher temperatures. This property was not exhibited by all fish to the same extent, but the muscles of those which were active formed ribose from added ribonucleic acid, adenosine triphosphate, ribonucleotides, ribosides and ribose 5-phosphate (Tarr, 1953, 1954*a*). The total pentose content of the muscles of certain marine fishes examined has ranged from 0·1 to 0·3 %, but it is not as yet known whether this occurs as free nucleic acid, as nucleic acid loosely combined with protein as in nucleotropomyosin (Hamoir, 1951*a*, *b*) or in some other form. Since nucleosides have not been observed to be present in appreciable amounts in muscles of fish which have been frozen in liquid nitrogen while still alive, but are often present in relatively high concentrations in such tissue *post mortem*, it would seem probable that they arise by enzymic degradation of nucleic acid. This suggests that fish muscles contain a ribonuclease and other enzymes which hydrolyse ribonucleic acid to its constituent mononucleosides.

The formation of free ribose from these mononucleosides could occur by their direct hydrolysis by riboside hydrolase enzymes, or by nucleoside phosphorolysis followed by hydrolysis of the ribose 1-phosphate formed. Riboside hydrolase enzymes have not often been described, phosphorylative mechanisms apparently occurring with greater frequency (Laskowski, 1951). However, Carter (1951) isolated a specific non-phosphorolytic uridine nucleosidase from yeast, and Lampen & Wang (1952) obtained both purine and pyrimidine hydrolytic nucleosidases from *Escherichia coli*. Ribose was not formed from either ribose 1-phosphate or ribose 5-phosphate by these two last-named preparations. The present experiments, which have already been described briefly (Tarr, 1954*a*), have shown that fish muscles contain non-phosphorolytic nucleosidases.

EXPERIMENTAL

Materials. The nucleosides, ribose, barium ribose 5-phosphate and yeast ribose nucleic acid were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, or from Schwarz Laboratories, Mount Vernon, New York. Cation exchange resin XE 64, a finely ground form of IRC 50, was purchased from Rohm and Haas Co., Philadelphia, Pennsylvania. The resin was washed and suspended in 0·2 M sodium phosphate buffer as described by Hirs, Moore & Stein (1953).

Paper electrophoresis. The technique used was practically identical with that described by Kunkel & Tiselius (1951). Enzyme preparations were freeze-dried before dissolving in 0·1 M veronal buffer (pH 8·6) for application in amounts not exceeding 0·05 ml. to Munktell 20S paper. The conditions used were a 16 hr. development at about 3° and 6 v/cm. The papers were stained with bromophenol blue and eluted into 0·01 N-NaOH as outlined by the above investigators.

Paper chromatography. Ribose was determined quantitatively by paper chromatography (Tarr, 1954*b*). Five separate 10 μl. amounts of reaction mixture were applied to Whatman no. 1 papers. The papers were developed 16 hr. at 20–22° with ethyl acetate–acetic acid–water solvent (3:1:3, by vol.) (Jermyn & Isherwood, 1949) and sprayed with aniline hydrogen phthalate reagent (Partridge, 1949). The above solvent separates D-ribose from other pentose sugars (Hochster & Watson, 1954), and it was found that the pentose formed from ribosides by the enzymes to be described had an R_F value identical with that of D-ribose. Nucleosides and their corresponding purine and pyrimidine bases were determined by quantitative paper chromatography (Paladini & Leloir, 1952). Five 5 μl. amounts were applied to Whatman no. 1 papers, which were developed for 3 hr. at 37° with freshly prepared ethyl acetate–acetic acid–water (3:1:1·75, by vol.) solvent. The R_F values were not

absolutely consistent with this system, but rarely varied more than $\pm 0{\cdot}02$ unit. The following R_F values were found: adenosine 0·6, adenine 0·68, guanosine 0·38, guanine 0·45, inosine 0·40, hypoxanthine 0·53, xanthosine 0·39, xanthine 0·50, cytidine 0·40, cytosine 0·51, uridine 0·56 and uracil 0·62. With guanine, xanthosine and xanthine, only a few micrograms could be applied to the paper, since overloading and consequent streaking tended to occur. Quantitative determinations were not made in these instances.

Chromatographic separation of ribose 1-phosphate and ribose 5-phosphate from ribose was carried out on Whatman no. 1 paper with *n*-butanol–acetic acid–water (1:1:0·5, by vol.) solvent. The enzyme reaction mixtures containing the pentose phosphate esters were applied in 5 μl. amounts to Whatman no. 1 paper, and were separated by ascending development with the above solvent for 8 hr. at 0°, at which temperature ribose 1-phosphate was not hydrolysed. With this system ribose 5-phosphate and ribose 1-phosphate had rather similar R_F values (0·31 and 0·37 respectively) and could be readily distinguished from ribose (R_F 0·5). The chromatograms were dried for 3 min. at 105° to remove the solvent, sprayed with aniline hydrogen phthalate reagent (Partridge, 1949) containing 0·5 ml. concentrated HCl/100 ml., and heated as usual.

Protein nitrogen. The method of Kingsley (1939) was used and was checked by micro-Kjeldahl determinations.

Enzyme preparation

Lingcod (*Ophiodon elongatus*) or rock cod (*Sebastodes* sp.) muscle from fish in rigor mortis was used. Muscle tissue which had been stored at −20° for several months also yielded satisfactory preparations. All procedures were conducted at about 0°. In a typical experiment 1·6 kg. of muscle were blended with 4·8 l. water and 204 g. of NaCl to bring the suspension to approximately 0·6 M with respect to this salt. N-HCl (240 ml.) was added slowly with rapid stirring to adjust the mixture to pH 4·6. The mixture on centrifuging at 2500 *g* yielded 4·7 l. of supernatant liquid. This was fractionated with $(NH_4)_2SO_4$ at pH 7·0. The fraction which precipitated between 0·4 and 0·6 saturation was collected by centrifuging, dissolved in water and dialysed for 2 days against repeated changes of demineralized water to yield 1·6 l. of crude riboside hydrolase containing 1 mg./ml. of protein N. Further purification was effected by treatment with cation-exchange resin XE64 as follows.

The crude enzyme (90 ml.) was mixed with 450 ml. of wet packed XE64 resin adjusted to pH 7·3, and the mixture kept for 30 min. at 0° with occasional stirring. The supernatant liquid was decanted, and the resin washed with fifteen successive 100 ml. portions of water by decantation. The enzyme preparation was eluted from the resin (about 450 ml.) by stirring in 66 g. NaCl (equivalent to about 2·5 M) gradually, and pouring the suspension on to a Whatman no. 4 conical filter. The resin was treated with 30 ml. of 2·5 M-NaCl and the surface then pressed firmly with a flattened glass rod to remove as much free liquid as possible. The eluate, after dialysing 18 hr. at 0° against several changes of demineralized water, measured 168 ml. and had 0·016 mg. protein N/ml. Analyses of the resin supernatants, washings and eluates in this and similar preparations showed that at least 95 % of the protein N initially present in the crude enzyme used could be accounted for. These resin eluates have been referred to as purified enzyme preparations. Neither crude nor purified enzyme preparations form ribose in absence of added nucleoside.

Experimental conditions. Unless otherwise stated, the nucleosides were used in 0·0133 M final concentration (equivalent to 2 mg./ml. of ribose), and were heated to effect solution where necessary. Xanthosine solutions were adjusted to pH 7. Enzyme reactions were carried out in small stoppered tubes containing 0·1 ml. each of nucleoside and buffer solutions and 0·2 ml. of enzyme preparation. The sodium phosphate and sodium acetate buffers used were 0·2 M with respect to the cations, and sodium veronal buffer 0·1 M with respect to diethylbarbituric acid.

RESULTS

Recovery and relative activities of enzyme preparations. The relative activity and recovery obtained with such preparations could not be assessed very accurately for there were considerable variations in their power to hydrolyse different ribosides. The calculations were also somewhat complicated by the facts that whole muscle contains undetermined amounts of ribosides, and its riboside hydrolase activity did not appear to be noticeably affected by pH, as is the case with the enzyme preparations. Thus with whole lingcod muscle, ribose formation with and without added inosine was found to be similar at natural pH (6·6) or at pH 5·7. The data given in Table 1 show that one purified preparation hydrolysed guanosine over 1500 times as actively on a protein basis as did whole muscle, but that the purification procedures, especially that involving the ion-exchange resin, resulted in very poor recoveries.

Electrophoresis experiments. Paper electrophoresis showed that crude riboside hydrolase preparations yielded two (and occasionally three) protein zones, while purified preparations yielded only a single zone which did not move appreciably from the point of application. This suggested that riboside hydrolase activity may be associated with a protein which does not migrate appreciably by either electrophoresis or electro-osmosis under the experimental conditions. However, although inosine was hydrolysed actively by preparations which had been applied to filter papers moistened with 0·1 M sodium veronal buffer pH 8·6, even after these had been stored several days at 0°, the enzyme activity was rapidly lost on the application of an electric potential (4–6 v/cm.). The reason for this loss of activity has not yet been established.

pH–*Activity relationship.* Crude preparations hydrolysed the four purine ribosides and cytidine, but not uridine, ribonucleic acid or ribonucleotides, at pH 5·6. On the other hand, at pH 8·6 inosine was the only riboside which was hydrolysed appreciably, though some crude preparations, which contained an active adenosine deaminase, also formed ribose from this substrate. The results obtained in a typical experiment are shown in Table 2.

Table 1. *Comparative activity and recovery of riboside hydrolases*

Blended whole muscle (3 g.) at natural pH 6·6 + nucleoside solution or water (1 ml.) formed the following amounts of ribose in 1 hr. at 37°: control, 220; guanosine, 290 and inosine, 620 μg./g. (1 g. ≃ 26 mg. protein N). The crude enzyme (0·5 mg. protein N/ml. final concentration) formed 1700 μg./ml. of ribose from guanosine and 1000 μg./ml. from inosine in 3 hr. at 37° in 0·1 M acetate buffer pH 5·0. The freeze-dried purified preparation (0·04 mg. protein N/ml.) formed 1070 μg./ml. of ribose from guanosine and 1060 μg./ml. from inosine in 3 hr. at 37° in 0·1 M acetate buffer pH 5·5, while the other purified preparation (0·005 mg. protein N/ml.) formed 520 μg./ml. of ribose from guanosine in 6 hr. under similar conditions. Neither crude nor purified enzyme preparations formed ribose in absence of added nucleosides.

Preparation studied	Ribose (μg.) formed in 1 hr. at 37° by 1 mg. of protein N*		Ribose (mg.) formed by 100 g. of original muscle or by the quantity of enzyme recovered from that amount of muscle†	
	Inosine	Guanosine	Inosine	Guanosine
Muscle	23·8	11·1	38·8	82·9
Crude enzyme	666 (28)	1134 (102)	28·4 (73)	16·6 (20)
Purified enzyme (freeze-dried)	8830 (370)	8930 (805)	4·2 (10·8)	4·12 (4·97)
Purified enzyme (not freeze-dried)	—	17 400 (1582)	—	4·70 (5·67)

* The figures in parentheses refer to the increase in activity in terms of that of the whole muscle.

† The figures in parentheses denote the actual percentage of the initial riboside hydrolase activity of the muscle which could be accounted for in given enzyme preparations.

Table 2. *Hydrolysis of ribosides at different* pH *values by a crude enzyme preparation*

Conditions: 0·46 mg./ml. protein N; 0·1 M phosphate buffer pH 5·6 or 0·05 M veronal buffer pH 8·6; incubation temperature 37°.

	Ribose (μg./ml.)			
	Initial pH 5·6		Initial pH 8·6	
	1 hr.	2 hr.	1 hr.	2 hr.
Adenosine	490	820	50	110
Guanosine	800	1130	50	50
Inosine	520	850	380	1020
Xanthosine	230	180	0	0
Cytidine	160	175	0	0

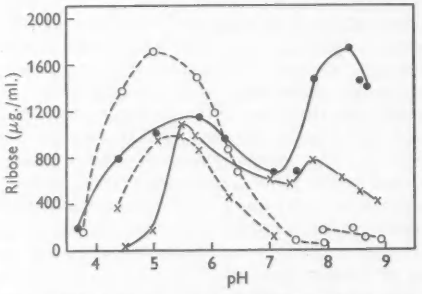

Fig. 1. pH/Activity curves of riboside hydrolase preparations with guanosine and inosine as substrates. Crude enzyme, inosine (●—●); purified enzyme, inosine (×—×); crude enzyme, guanosine (○– – –○); purified enzyme, guanosine (×– – –×). Crude enzyme, not freeze-dried, 0·5 mg. protein N/ml.; purified enzyme, freeze-dried, 0·04 mg. protein N/ml. Incubation period 3 hr. at 37°. Buffers: pH 3·7–5·5, 0·1 M acetate; pH 5·5–7·8, 0·1 M phosphate; pH 7·8–9·0, 0·05 M veronal.

Further experiments with crude and purified preparations confirmed and extended these initial findings. Fig. 1 shows the pH/activity curves which were obtained when crude and purified enzyme preparations hydrolysed guanosine and inosine. Guanosine was hydrolysed most actively at pH 5·0 by the crude preparations, and at pH 5·5 by the purified enzyme preparations. No hydrolysis of this riboside occurred at pH values above 7 with the purified enzyme, though the crude enzyme exhibited slight activity in this region. With inosine as substrate both the crude and purified preparations exhibited two pH optima, one in the acid and the other in the alkaline region. This indicated that the purified riboside hydrolase preparations possessed two distinct enzymes, one which hydrolysed inosine with a pH optimum of about 8, and another which hydrolysed purine ribosides and cytidine and having a pH optimum of about 5·5. With purified enzyme preparations hydrolysis proceeded at approximately the same rate in phosphate or acetate buffers at pH 5·5, and in veronal or phosphate buffers at pH 8·0, and it therefore was concluded that the type of buffer had no important effect on the pH–activity relationship.

Substrate specificity. The substrate specificity of purified enzyme preparations was studied at both acid and alkaline pH optima. Lingcod muscle itself hydrolyses uridine (Tarr, 1954*b*), and since neither crude nor purified enzyme preparations from this source do this, it would seem likely that the muscle possesses a uridine hydrolase which is distinct from the present enzymes. The results of an experiment in which single ribosides and mixtures of ribosides were hydrolysed with a purified enzyme preparation at pH 5·5 are given in Table 3. They

Table 3. *Ribose formation from single ribonucleosides and from mixtures containing equal amounts of two ribonucleosides at* pH 5·5

Protein N 0·033 mg./ml.; 0·1 M acetate buffer pH 5·5; 3 hr. incubation at 37°.

Substrate	Ribose concentration in nucleosides (mg./ml.)	Ribose formed (μg./ml.)	Theoretical amount of ribose assuming that both ribosides were hydrolysed independently (μg./ml.)
Adenosine	2	490	—
Adenosine	4	620	—
Inosine	2	470	—
Inosine	4	580	—
Guanosine	2	600	—
Guanosine	4	790	—
Xanthosine	2	150	—
Xanthosine	4	160	—
Cytidine	2	100	—
Cytidine	4	160	—
Uridine	2	0	—
Uridine	4	0	—
Adenosine + inosine	4	570	960
Adenosine + guanosine	4	510	1090
Adenosine + xanthosine	4	510	640
Adenosine + cytidine	4	505	590
Adenosine + uridine	4	360	490
Inosine + guanosine	4	570	1070
Inosine + xanthosine	4	500	620
Inosine + cytidine	4	500	570
Inosine + uridine	4	280	470
Guanosine + xanthosine	4	520	750
Guanosine + cytidine	4	660	700
Guanosine + uridine	4	560	600
Xanthosine + cytidine	4	180	250
Xanthosine + uridine	4	170	150
Cytidine + uridine	4	50	100

Table 4. *Ribose formation from single ribonucleosides and from mixtures containing equal amounts of two ribonucleosides at* pH 8·6

Protein N 0·063 mg./ml.; 0·1 M veronal buffer pH 8·6; 4 hr. incubation at 37°.

Substrate	Ribose concentration in nucleoside(s) (mg./ml.)	Ribose formed (μg./ml.)
Adenosine	2	0
Adenosine	4	0
Inosine	2	260
Inosine	4	350
Guanosine	2	0
Xanthosine	2	0
Cytidine	2	0
Uridine	2	0
Inosine + adenosine	4	< 25
Inosine + guanosine	4	255
Inosine + xanthosine	4	275
Inosine + cytidine	4	260
Inosine + uridine	4	245

indicate that nucleoside hydrolysis in the acid pH range is probably due to a single enzyme since in no instance did a mixture of two different nucleosides yield the amount of ribose which would be expected if separate enzymes were involved. In fact, in almost every instance the amount of ribose formed from a mixture of two ribonucleosides was slightly less than that formed from any single riboside at the 4 mg./g. ribose equivalent level. The existence of this competitive effect, and lack of an additive one, would lend support to the assumption that the enzyme which has a pH optimum of about 5·5 is a non-specific purine and pyrimidine nucleoside hydrolase.

The substrate specificity of the enzyme which had a pH optimum of about 8·0 was quite different. It hydrolysed only inosine, and this hydrolysis was almost completely inhibited by adenosine, and not by other purine ribosides (Table 4). It is therefore a specific inosine hydrolase.

Temperature–activity relationship. Fish muscle acid riboside hydrolase is active over a wide range of temperatures. The action was comparatively slow between 0 and 20°, but increased markedly above 20° as is shown in Table 5. At 50 and 55° hydrolysis was very rapid but the enzyme soon became inactive.

Proof that direct hydrolysis of ribosides occurs. Purified riboside hydrolase was incubated with various ribosides at pH 5·5, and the simultaneous appearance of both free ribose and the corresponding bases was determined qualitatively and, in several

instances, quantitatively by paper chromatography. The qualitative tests showed that hydrolysis of a given ribonucleoside was accompanied by simultaneous formation of free ribose and the corresponding purine or pyrimidine base. Table 6 shows that the amount of ribose which appeared on hydrolysis was, in those instances tested, about equivalent to that which was calculated from the quantity of purine or pyrimidine formed.

A purified freeze-dried riboside hydrolase preparation (0·06 mg. protein N/ml.) was incubated 3 hr. at 37° in 0·05M veronal buffer pH 8·6 or in 0·1M acetate buffer pH 5·5 containing 2 mg./ml. of ribose 1-phosphate or ribose 5-phosphate, or 4 mg./ml. of inosine. The reaction mixtures were analysed for ribose and for the ribose phosphates by paper chromatography. The preparation hydrolysed inosine at pH 5·5 (2200 μg./ml. of ribose) and at pH 8·6 (590 μg./ml. of ribose). However, no ribose could be detected when amounts of reaction mixtures equivalent to 20 μg. of ribose 1-phosphate or ribose 5-phosphate were chromatographed at 0° as described above. Since the reagent used to spray the chromatogram allows the detection of 0·25 μg. of ribose, it was concluded that purified riboside hydrolase does not hydrolyse either of the ribose phosphate esters. This indicates that the enzyme is a true hydrolase and that phosphorolysis of the ribosides is not involved. The fact that purified preparations which have been thoroughly dialysed against demineralized water hydrolyse ribosides equally well in acetate or phosphate buffer of the same pH values also indicates that these enzymes are strictly nucleoside hydrolases.

Isolation of D-*ribose and xanthine.* Guanosine (500 mg.) was incubated at 37° with 40 ml. of crude riboside hydrolase containing 1·5 mg. protein N/ml. The mixture was adjusted to pH 5·0 with 0·2N-HCl and was stirred gently, the pH being kept between 5·0 and 5·5 by regular addition of HCl. After 10 hr. the pH did not rise appreciably, and quantitative chromatographic analysis indicated that approximately the theoretical amount of ribose was present (265 mg.). The mixture was brought to pH 4·6, chilled to 0°, and the precipitate of protein and purine base removed by centrifuging at 0° and 14000 ***g***. The precipitate was suspended in 20 ml. of water and the centrifuging repeated. The clear supernatant fluids were pooled, freeze-dried, and the dry residue extracted by shaking mechanically with four successive 25 ml. portions of 99% ethanol. The clear filtrate was dried *in vacuo* over H_2SO_4 and ribose diphenylhydrazone prepared from the residue by the method of Mandl & Neuberg (1952). Crude ribose diphenylhydrazone (364 mg., m.p. 142°) was obtained in 65% of the theoretical yield. The crude material was recrystallized from hot chloroform and its characteristics were compared with those of similarly crystallized ribose diphenylhydrazone prepared from an authentic sample of D-ribose. (Mixed m.p. and m.p. of authentic and unknown 137–138°; C, 64·66; H, 6·50. Calc. for $C_{17}H_{20}N_2O_4$: C, 64·54, H, 6·37%. The following values were found for $[\alpha]_D^{22}$ in methanol.

Table 5. *Temperature–activity relationships of a purified riboside hydrolase preparation*

0·044 mg. protein N/ml.; 0·05M phosphate buffer pH 5·6.

	Ribose (μg./ml.)					
	Guanosine			Inosine		
Temp. (°)	1 hr.	2 hr.	4 hr.	1 hr.	2 hr.	4 hr.
20	50	—	120	60	—	140
25	140	—	300	185	—	200
30	145	—	500	180	—	310
37	180	460	1120	—	230	580
45	270	610	920	—	330	570
50	310	560	—	—	400	—
55	500	505	—	220	505	—

Table 6. *Hydrolysis of ribosides with simultaneous liberation of purine or pyrimidine base and of ribose*

Ribosides 0·0266M, purified enzyme 0·1 mg. protein N/ml., 0·1M acetate buffer pH 5·5, 37°.

		Corresponding	Ribose	
Substrate	Time (hr.)	base found (μg./10 μl.)	Found (μg./10 μl.)	Calc. (μg./10 μl.)
Adenosine	1	6·97	8·00	7·75
	2	9·53	12·68	10·6
	4	23·7	30·4	26·3
Inosine	1	3·68	4·24	4·05
	2	14·8	15·2	16·3
Xanthosine	2	8·2	7·10	8·09
Cytidine	2	7·7	8·00	11·4

Table 7. *Stability of freeze-dried riboside hydrolase*

Enzyme corresponded to 2 ml. purified preparation. To this was added 0·2 ml. inosine (equiv. to 4 mg./ml. ribose) and 0·2 ml. 0·2M acetate buffer pH 5·5. 2 hr. incubation at 37°.

Storage conditions		Ribose formed from inosine
Days	Temp. (°)	(μg./ml.)
1	−20	1180
1	0	1190
1	20	1050
18	−20	1110
18	0	1130
18	37	1035

Authentic: initial, −3·81°; 24 hr., −7·27°; 48 hr., −3·1°. Unknown: initial, −5·37°; 24 hr., −7·70°; 48 hr., −3·8°.)

The residue from the ethanol extraction was shaken several hours at room temperature with 10% NH_4OH (v/v) and the insoluble residue removed by centrifuging. The supernatant liquid was evaporated *in vacuo* over H_2SO_4 to yield 280 mg. of a dry, white residue, which had an absorption spectrum in the ultraviolet region similar to that of xanthine. The purity was only 55% as calculated from the molar extinction coefficient in 0·1N-NaOH at 285 mμ. This represented 57% of the theoretical amount, assuming both complete hydrolysis of the guanosine and deamination of the guanine formed. The crude material was stirred for 1 hr. with 25 ml. N-HCl, filtered with suction and the filtrate adjusted to pH 7 with 2·5N-NaOH. The white crystalline material which formed on standing overnight at 0° was collected on a sintered glass filter, washed with water, and dried over P_2O_5 *in vacuo* (yield 120 mg.). This material, when dissolved in 0·1N-NaOH, had an ultraviolet extinction coefficient at 285 mμ. characteristic of xanthine. $\epsilon_{calc.}^{10^{-4}}$ 0·99, found 0·92 (93%) (Stimson & Reuter, 1943, Hotchkiss, 1948). The following absorption ratios were found: 290/280 = 0·87, 250/260 = 1·08, 280/250 = 1·96; literature values for xanthine 0·91, 1·12 and 2·04 respectively (Hotchkiss, 1948).

Stability of freeze-dried preparations. A purified preparation which had been dialysed 18 hr. against demineralized water and which was not quite free from sodium chloride was freeze-dried in 2 ml. portions in small vials which were sealed and stored at four different temperatures. The results of activity tests (Table 7) indicate that freeze-dried preparations are fairly stable for short periods. No prolonged storage tests have yet been made. Tests indicate that the presence of a trace of NaCl in these preparations improves their stability.

SUMMARY

1. A method of preparation of crude riboside hydrolase enzymes from muscles of certain marine fishes is described. The muscle is extracted with cold 0·5–0·6M-NaCl or KCl at pH 4·6 and the extract fractionated with $(NH_4)_2SO_4$ at pH 7, the fraction obtained between 0·4 and 0·6 saturation possessing most of the activity.

2. Crude preparations were purified by stirring with Amberlite cation-exchange resin XE 64 at pH values slightly above 7, eluting with NaCl and dialysing.

3. Crude preparations contained two (and occasionally three) proteins as judged by zone electrophoresis while purified preparations showed a single protein zone.

4. The purest preparation obtained hydrolysed guanosine over 1500 times as actively as did whole muscle on a protein N basis.

5. Purified preparations contained two distinct enzymes, a non-specific riboside hydrolase with a pH optimum of about 5·5 which hydrolysed purine ribosides and cytidine, and a specific inosine hydrolase with a pH optimum of about 8·0. These enzymes were not separated. Neither ribose 1-phosphate nor ribose 5-phosphate was hydrolysed, and nucleoside hydrolysis proceeded in the absence of added inorganic phosphate.

6. D-Ribose diphenylhydrazone and xanthine were isolated from a reaction mixture of guanosine and crude riboside hydrolase.

7. The enzyme preparations withstood freeze-drying and were active even at 55°.

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Phosphorylation Coupled with the Oxidation of α-Ketoglutarate by Heart-Muscle Sarcosomes

3. EXPERIMENTS WITH FERRICYTOCHROME *C* AS HYDROGEN ACCEPTOR*

By E. C. SLATER
Molteno Institute, Downing Street, University of Cambridge

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Belitzer & Tsibakowa (1939) and Ochoa (1940, 1941) found that more than one atom of phosphorus is esterified for each atom of oxygen consumed by respiring tissue preparations, and concluded that the phosphorylation could not all be associated with the initial dehydrogenation of the substrate, but must occur also at some point in the respiratory chain of catalysts over which the hydrogen atoms of the substrate (or their electrons) pass on their way to oxygen. Ochoa's (1943) attempts to demonstrate phosphorylation in the respiratory chain directly by the use of a reduced hydrogen carrier, reduced diphosphopyridine nucleotide (DPNH), as a substrate were unsuccessful. Using improved techniques, Friedkin & Lehninger (1949) were able to obtain phosphorylation coupled with the oxidation of DPNH, and in later papers by Lehninger and his co-workers (Lehninger, 1949; Lehninger & Smith, 1949; Lehinger, 1951*a*), it was shown that in the oxidation of β-hydroxybutyrate to acetoacetate all the phosphorylation occurs in the reaction between DPNH and oxygen, and none between substrate and diphosphopyridine nucleotide.

Friedkin & Lehninger (1949) and Lehninger (1949) attempted to locate the phosphorylation steps in the respiratory chain more closely by experiments in which different parts of the chain were studied in isolation. In the first set of experiments, DPNH or β-hydroxybutyrate was used as the hydrogen donor with ferricytochrome *c* as the stoicheiometric hydrogen acceptor. In the second set of experiments, ferrocytochrome *c* was not used as a stoicheiometric hydrogen donor, but, instead, catalytic amounts of cytochrome *c* were used in the presence of different substances which directly reduce cytochrome *c* (Keilin & Hartree, 1938). No phosphorylation was obtained in the first set of experiments while, in the second, the only phosphorylation found was a small amount with ascorbic acid as the reducing agent. Lehninger was unable to draw any conclusions from these experiments concerning the localization of the phosphorylation in the respiratory chain.

Phosphorylation was first demonstrated with ferricytochrome *c* as the hydrogen acceptor in 1950, using α-ketoglutarate (α-oxoglutarate) as substrate, heart-muscle granules (sarcosomes, mitochondria) as the enzyme system and ferricytochrome *c*, in much smaller amounts than used by Lehninger, as hydrogen acceptor (Slater, 1950).

The P:O ratio (number of atoms of phosphorus esterified per oxygen atom equivalent of the ferricytochrome *c*) was 2·2, and there was reason to believe that this value might be underestimated. Since the P:O ratio with oxygen as hydrogen acceptor under the same conditions was 2·5, it was concluded that all the phosphorylation steps occurred between substrate and ferricytochrome *c*, implying that none occurred between ferrocytochrome *c* and oxygen.

This conclusion has been challenged on two grounds.

(1) Hunter (1951) and Krebs, Ruffo, Johnson, Eggleston & Hems (1953) have concluded, on the basis of thermodynamic calculations, that not more than three energy-rich phosphate bonds could be synthesized in the reaction between α-ketoglutarate and cytochrome *c* or more than one bond in the reaction between succinate and cytochrome *c*. Since these workers believe that the maximum P:O ratios in the aerobic oxidation of α-ketoglutarate and succinate are 4 and 2 respectively, it is argued that at least one phosphorylation must occur between cytochrome *c* and oxygen.

(2) Judah (1951) and Copenhaver & Lardy (1952) have found considerable esterification of inorganic phosphate during the oxidation of ascorbic acid by cytochrome *c* and liver mitochondria, suggesting that phosphorylation must occur in the cytochrome oxidase reaction. Nielsen & Lehninger (1954) have now reported phosphorylation with stoicheiometric amounts of ferrocytochrome *c* as hydrogen donor.

Lindberg & Ernster (1954) have made more specific criticisms of the earlier experiments (Slater, 1950).

The present paper describes the earlier experiment in greater detail, as well as additional experiments carried out under more strictly controlled conditions. These additional experiments support the validity of the previous measurements in demonstrating phosphorylation with ferricyto-

* Part 2, Slater & Holton (1954).

chrome *c* as hydrogen acceptor. However, they do not confirm the conclusion that the phosphorylation ratios with added ferricytochrome *c* as hydrogen acceptor is as high as with oxygen. Experiments of this type do not give a definite answer to the question of whether all phosphorylation occurs between substrate and ferricytochrome *c*.

METHODS

Sarcosomes

The suspensions of sarcosomes were prepared from cat, pig or rat heart, using the isolation media and the procedure described by Cleland & Slater (1953). Different types of preparations are designated according to the isolation medium.

Measurement of phosphorylation ratios with oxygen as hydrogen acceptor

The procedure adopted was that described by Slater & Holton (1954). Except where stated otherwise, the composition of the reaction mixture was as specified under 'standard conditions' in Table 10 of that paper.

Measurement of phosphorylation ratios with ferricytochrome c as hydrogen acceptor

Cytochrome c. Several different preparations of cytochrome *c* were used in this study. Some, made by the method of Keilin & Hartree (1945), contained 0·34% iron. Others, many of which were kindly supplied by Dr E. Margoliash, were purified by the procedure of Margoliash (1954) and contained 0·45% iron. Since the cytochrome *c* in these preparations was partially reduced, the concentration of ferricytochrome *c* was determined immediately before use by the following method. The optical density at 550 mμ. of 3 ml. of a suitably diluted solution in 0·05M phosphate buffer, pH 7·3, was measured (D_1) in a 1 cm. cell against a reference cell containing 3 ml. of the phosphate buffer alone. An excess of $K_3Fe(CN)_6$ (in 0·1 ml.) was added to each cell and the optical density again measured (D_2, after correction for the change of volume). A few crystals of dithionite (sufficient to decolorize the $K_3Fe(CN)_6$) were then added to each cell and the optical density was read a third time (D_3, after the same correction as above). The molar concentration of ferricytochrome *c* in the diluted sample equals $3{\cdot}62 \times 10^{-5} \times D_3(D_2 - D_1)/(D_3 - D_2)$.

The reduction of ferricytochrome *c* by α-ketoglutarate in the presence of the sarcosomes was followed spectrophotometrically in a Beckman or a Hilger Uvispek spectrophotometer. The concentration of cytochrome *c* was so great that the usual wavelength for studying cytochrome *c* (550 mμ., the position of the α-band) could not be used. In some experiments, the maximum wavelength of the β-band (520 mμ.) was used. In others, the decrease of the absorption at 580 mμ. on reduction was more convenient. The reoxidation of ferrocytochrome *c* was prevented either by adding cyanide or by using completely anaerobic conditions.

Cyanide experiment. The procedure adopted in the experiment previously reported (Slater, 1950) and summarized in Table 1 and Fig. 1 will be described in detail, although this does not include various improvements in technique which were introduced in later experiments.

Three Beckman cells (1·0 cm. light path) were filled as follows:

Beckman cell no. ...	1	2	3
Reaction mixture	+	+	+
Cytochrome *c*	+	+	+
α-Ketoglutarate	−	+	+
Final volume (ml.)	2·8	2·5	2·77

The composition of the reaction mixture is given in Table 1. Four conical flasks were filled as Beckman cell no. 3, except that in two flasks water replaced the cytochrome *c*. A few minutes before the experiment was begun, 0·2 ml. sarcosomal suspension and 0·1 ml. 0·15M-$MgCl_2$ were added to Beckman cell no. 1 and the optical density (at 520 mμ.) followed until it was constant. This cell was used as the reference for following the reduction of cytochrome *c* in cells nos. 2 and 3.

KCN (0·3 ml. of 0·1M) was added to Beckman cell no. 2, followed almost immediately, at zero time, by 0·1 ml. 0·15M-$MgCl_2$ and 0·2 ml. of the sarcosomal suspension. The optical density was followed (see curve I, Fig. 1) until a stationary reading was obtained. Trichloroacetic acid (0·5 ml. of 40%, w/v) was added at 8·2 min.

Beckman cell no. 3 was then treated in exactly the same way, except that only 0·03 ml. 0·1M-KCN was used. Trichloroacetic acid (TCA) was added to this cell after 7·6 min.

The solutions in the conical flasks were treated in the same way, except that the optical density changes were not followed. The times of addition of TCA are shown in Table 1.

Anaerobic experiments. Three types of apparatus were used to exclude oxygen.

(i) Manometric flask. This was used in only one experiment, the one described in the preliminary communication (Slater, 1950; see lines 6 and 7 of the table) and in Table 4 of the present paper. This experiment was carried out with the same preparation and at the same time as the cyanide experiment described above. A Warburg manometric flask with two side bulbs and a centre well was used. The main compartment contained the reaction mixture while 0·2 ml. of the sarcosomal preparation was placed in one side-bulb and 0·5 ml. 40% TCA in the other. The air in the flask was swept out with nitrogen and a stick of yellow phosphorus placed in the centre well. The flask was rapidly attached to a manometer and thoroughly flushed through with N_2 while being gently shaken. The taps were closed and the shaking continued for another 5 min. During all this period, the side bulb containing the sarcosomal preparation was immersed in ice. The sarcosomal preparation was then mixed with the reaction mixture in the main compartment and the manometer placed in a bath at 24°. After 8·5 min., the TCA was tipped into the main compartment to terminate the reaction. A second Warburg flask with the same solutions, except for the omission of the cytochrome *c*, was treated simultaneously in the same way.

It was not possible to observe accurately the completion of the reduction of the cytochrome *c* in this experiment. Since the cytochrome *c* was completely reduced in 5·6 min. in the presence of 0·001M-KCN (see Fig. 1), the 8·5 min. used in the anaerobic experiment included some margin of safety.

(ii) Side-arm Thunberg tubes. Thunberg tubes with a side-arm hollow stopper as well as the usual top hollow stopper have been described by Keilin & Hartree (1947*b*). The reaction mixture (2·8–2·9 ml.) was placed in the main tube, the sarcosomal preparation (0·1–0·2 ml.) in the side-arm stopper which could be immersed in an ice bath, while the top stopper contained 0·5 ml. 40% TCA. The tube was attached to a pump, and while vigorously shaken, evacuated sufficiently to make its contents boil, then filled with O_2-free nitrogen, and the whole process repeated twice. Finally, the tube was evacuated and the stopper closed. During all this treatment, the side-stopper containing the sarcosomes was immersed in ice. The reaction was commenced by mixing the contents of the side-stopper with the main tube and terminated by adding the TCA.

The completion of the reduction of ferricytochrome *c* could not be observed accurately in these tubes. The time required for complete reduction was determined in a separate experiment with the same preparation and reaction mixture, usually in the spectrophotometric Thunberg tube, and a few minutes were allowed as a margin of safety to ensure complete reduction.

(iii) Spectrophotometric Thunberg tubes. This was the most satisfactory method. The spectrophotometric Thunberg tubes (supplied by The Thermal Syndicate Ltd., 12–14 Old Pye Street, London, S.W. 1, under the name 'Beckman-Thunberg cells in vitreosil, 10 mm.') consist of a Thunberg tube sealed on to a 1 cm. silica cell, fitting into the cell-holder of the Beckman spectrophotometer. The tube projects above the top of the cell compartment and a special hood was constructed to prevent light entering the instrument. The shape of the cell makes it impossible to remove the dissolved O_2 by vigorous shaking during evacuation, and the following procedure was found necessary. The reaction mixture with all components except the hexokinase was placed in the main cell compartment of the apparatus and O_2-free nitrogen was gently bubbled through for about 30 min. The hexokinase (not more than 0·1 ml.) was added to the main tube, and the hollow stopper containing the enzyme inserted. The tube was then evacuated and filled with O_2-free nitrogen as described above, the evacuation and filling repeated twice and finally evacuated. The stopper containing the sarcosomes was kept in ice during all this period. At zero time, the sarcosomes were mixed with the reaction mixture in the main compartment and the tube placed in the spectrophotometer. Readings of optical density were made against a reference cell, open to the air, containing all the components of the reaction mixture, except the cytochrome *c*. The sarcosomal preparation was added to this reference cell immediately before zero time. When the optical density was steady (an example is given in Fig. 3, curve 1), the tube was quickly removed from the instrument and filled with O_2-free nitrogen. The stopper was removed and 0·5 ml. 40% (w/v) TCA added immediately. A negligible amount of O_2 entered the solution during this operation.

The amount of ferricytochrome *c* reduced could be calculated from the difference (ΔD_{580}) between the initial optical density (obtained by extrapolation) and the final reading, using the relationship, molar concentration of ferricytochrome *c* reduced $= 3{\cdot}62 \times 10^{-4} \Delta D_{580}$. The value calculated in this way agreed closely with the amount of ferricytochrome *c* added, measured as described above (p. 393). The latter value was considered the more reliable, and was used in the calculation of the P:O ratio. It was assumed that all the ferricytochrome *c* was reduced by the α-ketoglutarate in a reaction catalysed by the sarcosomes. Control experiments showed that there was no reduction of the ferricytochrome *c* by substances present in the reaction mixture, until the sarcosomes were added.

Measurement of phosphorylation

The aim of these experiments was to compare the yield of oxidative phosphorylation obtained with ferricytochrome *c* and with oxygen as hydrogen acceptors, under otherwise identical conditions. The reactions in the presence of yeast hexokinase and glucose are

$$\alpha\text{Kg} + A + x\text{ADP} + x\text{H}_3\text{PO}_4 \rightarrow \text{succinate} + \text{CO}_2 + A\text{H}_2 + x\text{ATP}, \quad (1)$$

$$x\text{ATP} + x\text{glucose} \rightarrow x\text{ADP} + x\text{HMP}, \quad (2)$$

where αKg = α-ketoglutarate, *A* = hydrogen acceptor (1 atom of oxygen or two molecules of ferricytochrome *c*), ADP = adenosine diphosphate, ATP = adenosine triphosphate, HMP = hexose monophosphate.

Two methods were used to determine the yields of esterified phosphate.

Method I. The amount of HMP found was corrected for the non-oxidative phosphorylation of glucose by ADP, catalysed by myokinase and hexokinase (reaction 3; AMP = adenosine monophosphate), as described by Slater (1953*b*)):

$$\text{ADP} + \text{glucose} \rightarrow \text{AMP} + \text{HMP}. \quad (3)$$

Method II. The increase of HMP + ~P (energy-rich phosphate) was measured, as described by Slater & Holton (1953). No correction for reaction 3 is necessary in method II, since (HMP + ~P) is not changed by this reaction.

An example of the use of method I is shown in Table 4. The 'myokinase correction' is here insignificant, amounting to only 2% of the ΔHMP. Examples of the use of method II are given in Tables 5–9.

It has been shown in an earlier paper (Slater & Holton, 1953) that method I is likely to give low values in short-time experiments, since it does not take account of the ATP which is synthesized by reaction 1, and has not had time to react according to reaction 2. In short-time experiments, this unreacted ATP is a considerable fraction of the total esterified phosphorus.

Analytical methods. Hexose monophosphate (HMP), energy-rich phosphorus (~P) and α-ketoglutarate (αKg) were measured as described previously (Slater, 1953*a*; Slater & Holton, 1953).

Hexokinase. The unit of hexokinase activity is that used by Berger, Slein, Colowick & Cori (1946).

RESULTS

Experiments using inhibitors of cytochrome oxidase

In experiments with ferricytochrome *c* as hydrogen acceptor, it is necessary to prevent the reoxidation of the ferrocytochrome *c*. One way of doing this is to inhibit the cytochrome oxidase. Cyanide is the most effective inhibitor, but in this case it suffers from the following disadvantages: (*a*) it inhibits phosphorylation; (*b*) owing to cyanohydrin formation, the concentration of free cyanide is much less than that added, so that there is an appreciable

Table 1. *Phosphorylation coupled with the reduction of ferricytochrome c by α-ketoglutarate. Cyanide experiment*

Fluoride, 0·039 M; phosphate, pH 7·3, 0·035 M; glucose, 0·016 M; AMP, 0·001 M; ADP, 0·001 M; Mg, 0·005 M; αKg, 0·0052* M; hexokinase; cat-heart sarcosomes (isolated in phosphate–saline), 1·06 mg. protein/ml. See Methods for procedure and Fig. 1 for course of reduction of ferricytochrome *c*. Temp., between 23·2 and 24·7°. Times corrected to correspond to 24·2°, assuming that the reaction velocity doubled for a rise in temp. of 10°.

ΔHMP, corrected = ΔHMP (in presence of cytochrome *c*) − ΔHMP (in absence of cytochrome *c*).

Time (min.)	5·6		7·9	10·2		8·2
HCN (total, M)	0·001		0·001	0·001		0·01
Ferricytochrome *c* (μatoms O equiv.)	0	0·23	0·23	0	0·23	0·23
HMP (μmoles): initial	0·16	0·16	0·16	0·16	0·16	0·16
final	0·22	0·52	0·66	0·29	0·79	0·40
Δ	0·06	0·36	0·50	0·13	0·63	0·24
ΔHMP, corrected (μmoles)	—	0·30	0·40†	—	0·50	0·22‡
P:O	—	—	—	—	2·20	—

* This value is somewhat less than that given previously (Slater, 1950), which was calculated on the assumption that the sample of α-ketoglutarate was 100% pure. The new value is based on an analysis of this sample.

† Assuming that ΔHMP in absence of cytochrome *c* = 0·10 (interpolated value).

‡ The value subtracted here was 0·015 μmole, found in an anaerobic experiment in the absence of cytochrome *c* (see Table 4).

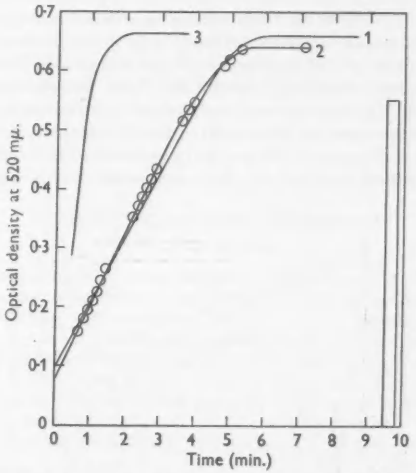

Fig. 1. Course of reduction of cytochrome *c* in the experiment described in Table 1. Curve 1: αKg, 0·01 M-KCN, 23·8°. Curve 2: αKg, 0·001 M-KCN, 24·7°. Curve 3 (shown for comparison): DPNH instead of α-ketoglutarate, 0·001 M-KCN, 24·2°. The actual optical density readings have been converted to correspond to a volume of 3·0 ml. The column to the right shows the optical density change corresponding to complete reduction of the cytochrome *c*. Individual measurements of optical density are shown for curve 2, only.

aerobic oxidation, which has to be allowed for; (*c*) it is not possible to estimate the disappearance of α-ketoglutarate during the reaction, since HCN in the TCA extracts causes the destruction of α-ketoglutarate; (*d*) HCN forms with ferricytochrome *c* a compound which does not react with the cytochrome *c*-reducing enzyme systems (Potter, 1941; Horecker & Kornberg, 1946); this means that the amount of ferricytochrome *c* actually reduced by the α-ketoglutarate is a little less than the total ferricytochrome *c*.

All these difficulties eventually led to the abandonment of this method of preventing the reoxidation of the ferrocytochrome *c*. However, it is necessary to refer to one experiment made by this method, since it has already been published (Slater, 1950). The results of this experiment are given in Table 1. The course of the reduction of the ferricytochrome *c* in the presence of both 0·01 and 0·001 M-HCN is shown in Fig. 1. Since method I was used to measure the phosphorylation, it is necessary to correct the ΔHMP found for the amount which would be synthesized by the combined action of myokinase and hexokinase. This was allowed for in control measurements made at the same time, in which the cytochrome *c* was omitted. These controls also allowed for the HMP synthesized by that fraction of the respiration which was not inhibited by the cyanide. This correction also takes account of any phosphorylation accompanying the dismutation of α-ketoglutarate (see p. 399).

It can be seen in Table 1 that the corrected values of ΔHMP continued to increase after all the ferricytochrome *c* was reduced (5·6 min.). This is due mostly to a lag in the synthesis of HMP from the ATP formed by the oxidative phosphorylation reaction (Slater & Holton, 1953). From this point of view, the highest value, corresponding to a P:O ratio of 2·2, is the nearest to the true value. This is practically identical with the value obtained (2·16) when the reoxidation of the

ferrocytochrome *c* was prevented by excluding oxygen (see Table 4).

However, the calculated value of 2·2 for the P:O ratio may be slightly high, since the control measurement made in the absence of cytochrome *c* is adequate only if the respiration in the presence of cyanide is unaffected by the addition of cytochrome *c*. Experiments made both with the Keilin & Hartree (1947*a*) heart-muscle preparation oxidizing succinate and with sarcosomal preparations oxidizing α-ketoglutarate showed that the respiration was slightly increased by the addition of cytochrome *c*, but the error introduced became serious only when a considerable fraction of the respiration was unaffected by the cyanide.

The differences between the initial (obtained by extrapolation) and the final optical densities in Fig. 1 show that the ferricytochrome *c* was virtually completely reduced. This shows that there must have been little respiration in this experiment. However, attempts to repeat this experiment with different types of preparations and reaction mixtures have failed, the optical density reaching a steady value (and then often declining again) when only about 70% of the cytochrome *c* was reduced. While the reason for this is not completely clear, it is probably connected with the fact that the reaction between α-ketoglutarate and cyanide is relatively slow. With high concentrations of active sarcosomal preparations and relatively low concentrations of ferricytochrome *c*, all the latter is reduced and the experiment is finished while the concentration of free cyanide is still sufficiently high to inhibit the cytochrome oxidase almost completely.

The extent of the combination between α-ketoglutarate and cyanide was determined in the following experiment, using a Keilin & Hartree heart-muscle preparation oxidizing succinate at 25°. A curve was constructed relating the rate of oxygen uptake to the concentration of cyanide. Another flask, containing 0·005 M α-ketoglutarate and $8{\cdot}75 \times 10^{-4}$ M total cyanide, consumed oxygen at a rate corresponding to a concentration of free cyanide of $5{\cdot}95 \times 10^{-5}$ M, i.e. 92·5% of the cyanide must have been bound by the α-ketoglutarate. The uniform rate of oxygen uptake showed that the reaction between the cyanide and α-ketoglutarate had come to equilibrium by the time the manometric readings commenced, about 20 min. after mixing the reactants.

The earlier stages of the reaction between α-ketoglutarate and cyanide were studied by a different type of experiment, which is described in Fig. 2. Curve 1 shows the rapid rate of aerobic oxidation of DPNH by the Keilin & Hartree heart-muscle preparation, and curve 2 shows the strong inhibition of this oxidation by $8{\cdot}75 \times 10^{-4}$ M cyanide. The addition of a small amount of water had no appreciable effect on this inhibition. The addition of α-ketoglutarate (curve 3), however, caused a steadily increasing release of the cyanide inhibition. Since the reaction between cyanide and cytochrome oxidase, and its reversal, is known to be a very rapid reaction, the slow release of the inhibition must be due to the slow reaction between α-ketoglutarate and cyanide. The reactivation was hardly detectable until about 3 min. after the addition of the α-ketoglutarate and did not become appreciable for several minutes later.

Inhibition of phosphorylation by cyanide. The last column of Table 1 shows that the phosphorylation is considerably less with 0·01 M-HCN than with 0·001 M-HCN (0·22 μmole HMP compared with 0·40 at approximately the same time). This suggests that cyanide is an inhibitor of oxidative phosphorylation. This was shown in another way in the experiment summarized in Table 2, in which varying concentrations of α-ketoglutarate were used in the presence of 0·001 M-HCN. Owing to cyanohydrin formation between α-ketoglutarate and cyanide, increasing the concentration of α-ketoglutarate has the effect of decreasing the concentration of cyanide, although this effect was probably lessened to some extent by the distillation of cyanide from the centre well to the main flask. Table 2 shows that with increasing α-ketoglutarate concentration, there was an increase both of the O_2 uptake and of the P:O ratio of the residual respiration. It should be noted that varying the concentration of α-keto-

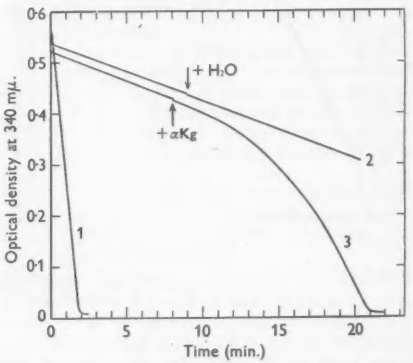

Fig. 2. The reaction between α-ketoglutarate and cyanide. The curves show the oxidation of DPNH by the Keilin & Hartree heart-muscle preparation. Phosphate, pH 7·4, 0·04 M; ethylenediaminetetraacetate, 0·001 M; DPNH, $8{\cdot}4 \times 10^{-4}$ M; heart-muscle preparation (0·49 mg. protein) added at zero time. Curve 1, no other addition; curve 2, $8{\cdot}75 \times 10^{-4}$ M-HCN initially present, 0·16 ml. water added at arrow; curve 3, $8{\cdot}75 \times 10^{-4}$ M-HCN initially present, 0·16 ml. α-ketoglutarate (15 μmoles) added at arrow. Optical densities corrected for change of volume after additions. Temp. 24–25°.

glutarate within this range has no appreciable effect on the P:O ratio in the absence of cyanide (Slater & Holton, 1954). The P:O ratio in the absence of cyanide was a little low in this experiment, probably because a rather small concentration of hexokinase (24 units/mg. sarcosomal protein) was used in order to decrease the possibility of the dismutation reaction, discussed below.

Effect of azide on oxidative phosphorylation. In view of the difficulties with cyanide, another inhibitor of cytochrome oxidase was sought. Sulphide could not be used because it rapidly reduces ferricytochrome *c* in the absence of any other enzyme. As was to be expected from Loomis & Lipmann's (1949) work, azide was unsuitable because it inhibited phosphorylation. Moreover, it was relatively ineffective as an inhibitor of the respiration of the sarcosomes. Both these effects are shown in Table 3. Since previous studies of the uncoupling action of azide on oxidative phosphorylation have been made only with ferricyanide as acceptor (Loomis & Lipmann, 1949; Judah, 1951), it is of some interest to show the same effect with oxygen as hydrogen acceptor. Table 3 shows that a concentration of azide which has no effect on the respiration markedly inhibits the phosphorylation. It was found in another experiment that azide, like dinitrophenol, does not completely uncouple the phosphorylation from respiration when α-ketoglutarate is substrate, i.e. azide is primarily an inhibitor of respiratory chain phosphorylation (cf. Judah, 1951). The P:αKg ratio with 0·003–0·015 M azide was about the same as with 1×10^{-4}–$2{\cdot}8 \times 10^{-4}$ M dinitrophenol (see Slater & Lewis, 1954).

It is surprising that the oxidation of α-ketoglutarate by the sarcosomes is so insensitive to azide. The oxidation of succinate is more sensitive, 85% inhibition being found with 0·03 M azide compared with 66% inhibition of the oxidation of α-ketoglutarate under the same conditions (Table 3). The rate of oxidation of succinate by the Keilin & Hartree preparation was inhibited by 92% by this concentration of azide. Except for the addition of succinate and the removal of malonate and fluoride, the reaction mixture in this experiment was the same as in Table 3 even to the extent of including α-ketoglutarate, which is not oxidized by the Keilin & Hartree preparation. Cyanide was equally inhibitory to both types of preparation oxidizing succinate.

Table 2. *Effect of cyanide on oxidative phosphorylation*

Rat-heart sarcosomes (isolated in 0·28 M sucrose, 0·01 M ethylenediaminetetraacetate, pH 7·4), 1·5 mg. protein/ml.; phosphate, pH 7·4, 0·03 M; sucrose, 0·045 M; malonate, 0·01 M; fluoride, 0·04 M; glucose, 0·03 M; ethylenediaminetetraacetate, 0·0015 M; AMP, 2×10^{-4} M; ADP, 2×10^{-4} M; Mg, 0·005 M; αKg, 0·01 M; no added cytochrome *c*; hexokinase, 24 units/mg. sarcosomal protein. The centre well of the manometric flask contained a solution prepared by mixing 0 ml. 2 M-KCN and 0·2 ml. 1 N-KOH. 30 min., 25°.

αKg (m-moles/l.) ...	1	3	10	10
HCN (m-moles/l.) ...	1	1	1	0
$-\Delta O$ (μatoms)	0·26	0·47	0·65	5·33
ΔHMP (μatoms)	0·22	0·60	1·35	13·0
P:O	0·84	1·28	2·08	2·43

Anaerobic experiments

The anaerobic experiment reported in the preliminary publication (Slater, 1950) is shown in greater detail in Table 4. There are a number of possible sources of error in experiments of this type, and these will now be considered in detail.

Residual oxygen. The most serious possible error in these experiments is the presence of residual oxygen. It is easy by repeated evacuation and refilling with oxygen-free nitrogen to remove practically all the oxygen from the gas phase, but special care is necessary to remove dissolved oxygen from the liquid. The reaction volume used (3 ml.) contains approx. 1·5 μatoms of dissolved oxygen in equilibrium with air. To remove this oxygen from the side-arm Thunberg tubes, it was found essential to shake the solution vigorously during the evacuation and filling. By the following method it was found that less than 0·01 μatom of oxygen then remained.

The main tube of a side-arm Thunberg tube contained a solution of ferrocytochrome *c* equivalent to 0·01 μatom of oxygen in phosphate buffer, total vol., 3·0 ml. The side stopper contained 0·1 ml. of a concentrated Keilin & Hartree heart-muscle preparation. In order to simulate conditions of the oxidative phosphorylation experiments, 0·5 ml. 40% (w/v) TCA was placed in the top stopper. After the usual evacuation procedure, the contents of the main

Table 3. *Effect of azide on oxidative phosphorylation*

Rat-heart sarcosomes (isolated in 0·28 M sucrose, 0·01 M ethylenediaminetetraacetate, pH 7·4), 0·74 mg. protein/ml.; phosphate, pH 7·4, 0·03 M; sucrose, 0·045 M; malonate, 0·01 M; fluoride, 0·04 M; glucose, 0·03 M; ethylenediaminetetraacetate, 0·0015 M; ADP, 6×10^{-4} M; AMP, 6×10^{-4} M; Mg, 0·005 M; αKg, 0·01 M; cytochrome *c*, $4{\cdot}9 \times 10^{-5}$ M; hexokinase, 292 units/mg. sarcosomal protein. 45 min., 25°.

Azide (M)	0	10^{-4}	5×10^{-4}	3×10^{-3}	$1{\cdot}5 \times 10^{-2}$	3×10^{-2}
$-\Delta O$ (μatoms)	3·13	3·07	3·34	2·10	1·20	1·05
ΔHMP + Δ~P (μmoles)	8·19	6·93	5·32	1·35	0·97	0·71
P:O	2·62	2·26	1·60	0·64	0·81	0·68

tube were mixed with the heart-muscle preparation and the suspension observed under a low-dispersion micro-spectroscope. The spectrum of ferrocytochrome *c* remained visible even after 24 hr. standing. When the experiment was repeated with different amounts of ferrocytochrome *c*, omitting the shaking during the evacuation (the solutions boiled vigorously), ferrocytochrome *c* equivalent to 0·10 μatom oxygen was completely and instantaneously oxidized when the solutions were mixed.

Table 4. *Phosphorylation accompanying the reduction of ferricytochrome c by α-ketoglutarate. Preliminary anaerobic experiment*

Anaerobic expt. using manometric flask; see p. 393 for procedure. Fluoride, 0·039 M; phosphate, pH 7·3, 0·035 M; glucose, 0·016 M; AMP, 0·001 M; ADP, 0·001 M; Mg, 0·005 M; αKg, 0·0052 M; hexokinase; cat-heart sarcosomes (isolated in phosphate–saline), 1·06 mg. protein/ml. 8·5 min., 24°.

Ferricytochrome *c* (μatoms O equiv.)	...	0·23	0
HMP (μmoles): initial		0·16	0·16
final		0·67	0·17
Δ		0·51	0·01
ΔHMP, corrected (μmoles)		0·50	—
P:O		2·16	—

The procedure used with the spectrophotometric Thunberg tubes (see p. 394) was also satisfactory. Residual oxygen can be detected in this case by a time lag in reaching the maximum rate of reduction of cytochrome *c*, as is clearly shown in Fig. 3. Curve 1 describes the course of the reduction of cytochrome *c* in the spectrophotometric Thunberg tube, the contents of which had been bubbled with oxygen-free nitrogen for some time before evacuation. Curve 2 describes an experiment with the same preparation, when the evacuation procedure was carried out without this preliminary bubbling treatment.

The anlyses on these two tubes are given in Table 5. The excess of $-\Delta\alpha$Kg over the oxygen equivalent of the ferricytochrome *c* (0·901 − 0·469 = 0·432) in tube 2 is a measure of the residual oxygen. If this residual oxygen phosphorylates with the same P:O ratio as in the aerobic experiment with the same preparation (2·8), then $2{\cdot}8 \times 0{\cdot}432 = 1{\cdot}21$ μatoms should be subtracted from the ΔHMP + $\Delta \sim$P found in this experiment. This gives $1{\cdot}97 - 1{\cdot}21 = 0{\cdot}76$ μatom P esterified by the reduction of ferricytochrome *c*, not significantly different from the 0·78 obtained in the other tube.

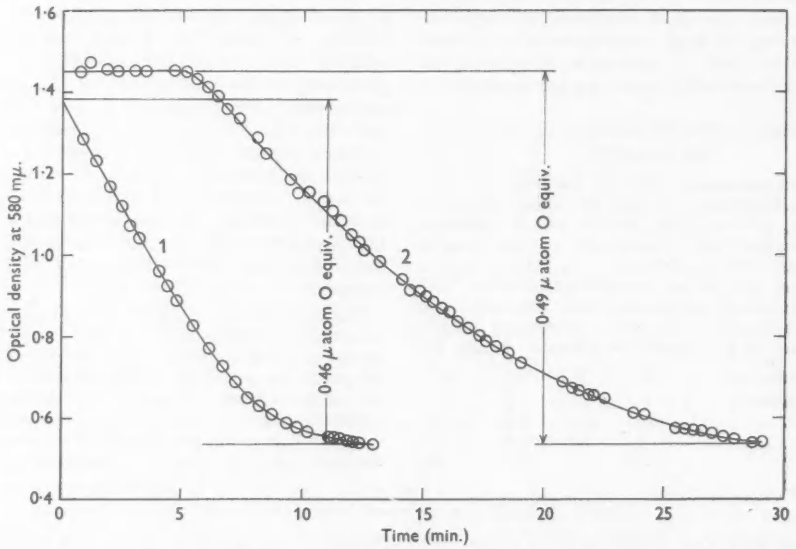

Fig. 3. Reduction of ferricytochrome *c* by α-ketoglutarate under anaerobic conditions and in presence of a small amount of oxygen. Rat-heart sarcosomes (isolated in 0·28 M-sucrose, 0·01 M ethylenediaminetetraacetate, pH 7·4), 0·47 mg. protein/ml.; phosphate, pH 7·4, 0·03 M; malonate, 0·01 M; fluoride, 0·04 M; AMP, 2×10^{-4} M; ADP, 2×10^{-4} M; αKg, 4×10^{-4} M; glucose, 0·02 M; ethylenediaminetetraacetate, 0·001 M; magnesium, 0·005 M; hexokinase, 151 units/mg. sarcosomal protein. Curve 1, tube completely evacuated; curve 2, tube contained residual oxygen. Ferricytochrome *c* equivalent to 0·47 μatom oxygen was used. The curves show the amount of ferricytochrome *c* reduced, calculated from the change of optical density.

This shows (*a*) that residual oxygen does not affect the phosphorylation obtained by the reduction of cytochrome *c*; (*b*) that even when there are large amounts of residual oxygen, its effect on the phosphorylation obtained with ferricytochrome *c* can be accurately allowed for.

Phosphomonoesterase. The sarcosomal preparations contain only small amounts of phosphomonoesterase which, under conditions of active and continuous oxidation, hydrolyse an insignificant proportion of the HMP synthesized. However, under the conditions of the anaerobic experiments, even a slight phosphomonoesterase activity will cause appreciable loss of HMP if the deproteinizing is delayed after the reduction of the cytochrome *c* is complete. Since some delay was inevitable, it was not possible completely to eliminate this source of error. However, only pig preparations contain sufficient phosphomonoesterase to cause a significant error. In the experiment described in Table 6, the reduction of ferricytochrome *c* was complete in about 4 min., but α-ketoglutarate continued to disappear, because of a reaction which will be considered below. Although this anaerobic reaction is accompanied by phosphorylation, so that in the absence of phosphomonoesterase the total esterified phosphorus would increase on standing, (ΔHMP + $\Delta\sim$P) actually decreased rapidly, owing to the action of phosphomonoesterase.

Dismutation of α-ketoglutarate. As already reported (Slater & Holton, 1954; Holton, 1954*a*), the yeast hexokinase preparations contain a substance which, when added to the sarcosomal preparations, promotes the disappearance of α-ketoglutarate in the absence of any other hydrogen acceptor. This reaction has been studied by Holton (1954*b*), who has found that it is accompanied by phosphorylation, with a P:αKg ratio of 0·5.

Even hexokinase purified to the stage of step 5 of the procedure of Berger *et al.* (1946) contains sufficient of this substance to promote the disappearance of considerable amounts of α-ketoglutarate, in the absence of oxygen or other hydrogen acceptor. Fortunately, this reaction proceeds at an appreciable rate only in the absence of other hydrogen acceptors, and in the experiments with ferricytochrome *c*, it can be made inconsiderable if the reaction is stopped soon after the reduction of the ferricytochrome *c* is complete. This is illustrated by the two experiments summarized in Tables 6 and 7, the former with crude hexokinase, the latter with the partially purified enzyme (specific activity, 20 % that of Berger *et al.*'s purified preparation). In both experiments, the disappearance of α-ketoglutarate was equivalent to the ferricytochrome *c* reduced, when the reaction was stopped shortly after the reduction was complete, but continued after all the ferricytochrome *c* was reduced. Tubes 6 and 7 of Table 7 show the very considerable anaerobic reaction in the absence of cytochrome *c*. The greater rate of a dismutation reaction in the absence of oxygen (or of cytochrome *c* which takes the place of

Table 5. *Effect of residual oxygen on the measurement of the phosphorylation accompanying the reduction of ferricytochrome c by α-ketoglutarate*

Anaerobic expt. using spectrophotometric Thunberg tube. See Fig. 3 for details.

Tube	1	2
Ferricytochrome *c* (μatoms O equiv.)	0·469	0·469
αKg (μmoles): initial	0·98	0·98
final	0·53	0·08
Δ	−0·45	−0·90
∼P (μmoles): initial	0·59	0·59
final	0·43	0·40
Δ	−0·16	−0·19
HMP (μmoles): initial	0·02	0·02
final	0·96	2·18
Δ	0·94	2·16
ΔHMP + $\Delta\sim$P	0·78	1·97
P:O	1·67	—
P:αKg	1·74	2·19

Table 6. *Measurement of phosphorylation coupled with reduction of ferricytochrome c by α-ketoglutarate. Effect of phosphomonoesterase in pig-heart sarcosomal preparations and of crude hexokinase*

Pig-heart sarcosomes (isolated in phosphate–saline), 1·3 mg. protein/ml.; phosphate, pH 7·3, 0·035 M; malonate, 0·01 M; fluoride, 0·04 M; AMP, $4{\cdot}8\times10^{-4}$ M; ADP, $4{\cdot}5\times10^{-4}$ M; αKg, $2{\cdot}8\times10^{-4}$ M; glucose, 0·017 M; Mg, 0·005 M; hexokinase, 127 units/mg. sarcosomal protein. Crude hexokinase. Side-arm Thunberg tubes used. Ferricytochrome *c* completely reduced in about 4 min., 22°.

Tube	1	2	3	4	5	6
Time (min.)	0	4	5	10	30	30
Ferricytochrome *c* (μatoms O equiv.)	0·30	0·30	0·30	0·30	0·30	0
αKg (μmoles)	0·84	0·57	0·43	0·23	0·04	0·04
$-\Delta$αKg (μmoles)	—	0·27	0·41	0·61	0·80	0·80
∼P (μmoles)	1·36	1·06	—	0·69	0·47	0·42
$-\Delta\sim$P (μmoles)	—	0·30	0·35*	0·67	0·89	0·94
HMP (μmoles)	0·05	0·89	1·01	1·10	1·09	0·89
ΔHMP (μmoles)	—	0·84	0·96	1·05	1·04	0·84
ΔHMP + $\Delta\sim$P	—	0·54	0·61	0·38	0·15	−0·10

* Interpolated value.

Table 7. *Measurement of phosphorylation coupled with reduction of ferricytochrome c by α-ketoglutarate. Correction for dismutation*

Rat-heart sarcosomes (isolated in 0·28 M sucrose, 0·01 M ethylenediaminetetraacetate, pH 7·4), 0·84 mg. protein/ml.; phosphate, pH 7·4, 0·03 M; malonate, 0·01 M; fluoride, 0·04 M; AMP, 2×10^{-4} M; ADP, 2×10^{-4} M; αKg, $3{\cdot}2 \times 10^{-4}$ M; glucose, 0·03 M; ethylenediaminetetraacetate, 0·0017 M; Mg, 0·005 M; hexokinase, 132 units/mg. sarcosomal protein. Cytochrome *c* completely reduced in approx. 9 min. Temp., 21°. Partially purified hexokinase used. Tube 2 was spectrophotometric Thunberg; Tubes 3 to 7 were side-arm Thunberg tubes.

Tube	1	2	3	4	5	6	7
Time (min.)	0	11	15	15	15	15	15
Ferricytochrome *c* (μatoms O equiv.) (*a*)	—	0·50	0·50	0·50	0·50	0	0
αKg (μmoles)	0·97	0·51	0·34	0·44	0·37	0·40	0·35
$-\Delta$αKg (μmoles) (*b*)	—	0·46	0·63	0·53	0·60	0·57	0·62
$\sim$P (μmoles)	0·59	0·58	0·57	0·58	0·59	0·61	0·59
$\Delta\sim$P (μmoles) (*c*)	—	−0·01	−0·02	−0·01	0	0·02	0
HMP (μmoles)	0·03	0·90	1·01	0·94	0·96	0·41	0·40
ΔHMP (μmoles) (*d*)	—	0·87	0·98	0·91	0·93	0·38	0·37
ΔHMP $+\Delta\sim$P (μmoles) ($c+d$)	—	0·86	0·96	0·90	0·93	0·40	0·37
($b-a$)	—	—	0·13	0·03	0·10	—	—
0·5 ($b-a$)	—	—	0·06	0·01	0·05	—	—
Corrected esterified P ($c+d$) − 0·5 ($b-a$)	—	0·86	0·90	0·89	0·88	—	—

Table 8. *Measurement of phosphorylation coupled with reduction of ferricytochrome c by α-ketoglutarate. Comparison of vacuum with nitrogen gas phase*

Rat-heart sarcosomes (isolated in 0·28 M sucrose, 0·01 M ethylenediaminetetraacetate, pH 7·4), 0·60 mg. protein/ml.; phosphate, pH 7·4, 0·03 M; malonate, 0·01 M; fluoride, 0·04 M; AMP, 2×10^{-4} M; ADP, 2×10^{-4} M; αKg, 4×10^{-4} M; glucose, 0·02 M; ethylenediaminetetraacetate, 0·001 M; Mg, 0·005 M; hexokinase, 116 units/mg. sarcosomal protein. Spectrophotometric Thunberg tubes. Tube 1 was finally evacuated; Tube 2 was finally filled with nitrogen.

Tube		1	2
Ferricytochrome *c* (μatoms O equiv.)		0·464	0·464
αKg (μmoles):	initial	1·10	1·10
	final	0·68	0·60
	Δ	−0·42	−0·50
$\sim$P (μmoles):	initial	0·62	0·62
	final	0·43	0·43
	Δ	−0·19	−0·19
HMP (μmoles):	initial	0·02	0·02
	final	1·00	1·12
	Δ	0·98	1·10
ΔHMP $+\Delta\sim$P		0·79	0·91
P:O		1·70	—
P:αKg		1·89	1·82

oxygen in this experiment) is well-known in biochemistry and can be explained by the competition between two pathways of hydrogen-transfer.

The contribution of the anaerobic reaction was allowed for by subtracting 0·5 ($-\Delta$αKg − oxygen equiv. of ferricytochrome *c*) from the measured phosphorylation (see Table 7). The correction was a small one, which could be accurately assessed.

Effect of evacuation procedure on activity of sarcosomes. The sarcosomal suspension in the evacuated spectrophotometric Thunberg tube often scatters less light than the identical suspension in an open spectrophotometric cell. Since Cleland (1952) has shown that swelling of the sarcosomes is associated with decreased light scatter, it is possible that the evacuation procedure causes the sarcosomes to swell. It was important to determine whether this swelling was sufficient to impair the phosphorylative activity of the sarcosomes. This was investigated in two experiments.

In the first, a side-arm Thunberg tube containing the usual reaction mixture without any cytochrome *c* was subjected to the usual evacuation procedure, except that some oxygen (0·25 μatom) was allowed to remain. The P:αKg ratio was 2·49, only slightly lower than the P:O ratio obtained in the usual way with this preparation (2·64). This indicates that the evacuation procedure does not appreciably affect the phosphorylative activity of the sarcosomes, when oxygen is the hydrogen acceptor.

This was confirmed in a second experiment, in which the top stopper was closed not after the fourth evacuation as usual but after a further filling with nitrogen. This experiment is summarized in Table 8, which shows that the P:αKg ratio was no higher when the tubes were finally filled with nitrogen instead of being left evacuated. Tube 2 of Table 8 shows why evacuation is preferable; the nitrogen, even after passage through heated copper, contains a measurable amount of oxygen.

Effect of concentration of ADP. When method II (p. 394) is used to measure the phosphorylation, it is not possible to increase the concentration of ADP beyond about 2×10^{-4} M, without introducing appreciable errors in the measurement of $\Delta\sim$P. This concentration of ADP is sufficient for optimum

aerobic phosphorylation (Slater & Holton, 1953), but it was necessary to check that this was also the case with ferricytochrome *c* as hydrogen acceptor. It was found that increasing the concentrations of ADP fivefold (using method I to measure the phosphorylation) had no effect on the phosphorylation (0·57 compared with 0·55 μmole).

Effect of hypotonic pretreatment of the sarcosomes. Lehninger (1951*a*, *b*) has found that the rate of oxidation of DPNH by liver mitochondria was greatly increased by previously exposing the mitochondria to hypotonic conditions. The P:O ratio was also increased by this hypotonic pretreatment.

Table 9 shows that, although suspension of the sarcosomes in a hypotonic medium nearly trebled the rate of reduction of ferricytochrome *c* by α-ketoglutarate and slightly increased the rate of aerobic oxidation, the phosphorylation ratios were unaffected. The rate of oxidation of ascorbate, and especially the accompanying phosphorylation, were greatly increased by this treatment. The phosphorylation with ascorbate will be discussed in a separate paper.

Effect of 2:4-dinitrophenol. It is now well-established that one of the phosphorylation steps accompanying the oxidation of α-ketoglutarate to succinate is substrate-linked, i.e. it is associated with the initial dehydrogenation of the substrate, and that this substrate-linked phosphorylation is insensitive to dinitrophenol (see Slater & Lewis, 1954, for a review of the literature). It was of interest, therefore, to determine the effect of dinitrophenol on the phosphorylation coupled with the reduction of ferricytochrome *c*. An experiment showing this is summarized in Table 10.

It can be seen that dinitrophenol markedly lowers the P:αKg ratios both with oxygen and with ferricytochrome *c* as hydrogen acceptor, and the P:αKg ratios in the presence of dinitrophenol are practically the same with both hydrogen acceptors. This is a good indication that the P:αKg ratio can be measured with ferricytochrome *c* as hydrogen acceptor without appreciable errors. The reason why the P:αKg ratio in the presence of dinitrophenol is less than 1 is discussed by Slater & Lewis (1954).

Comparison of phosphorylation ratios with oxygen and ferricytochrome c as hydrogen acceptors

Table 11 summarizes all the experiments made with ferricytochrome *c* as hydrogen acceptor in the absence of oxygen, in which normal sarcosomal preparations were used and there was close agreement between the α-ketoglutarate disappearance and the ferricytochrome *c* used, showing the absence of residual oxygen and of the anaerobic dismutation of α-ketoglutarate. Two experiments were also carried out with preparations made in

Table 9. *Effect of hypotonic pretreatment on rate of oxidation of α-ketoglutarate by ferricytochrome c and by oxygen, on rate of oxidation of ascorbate by oxygen and on phosphorylation ratios*

Rat-heart sarcosomes isolated in 0·21 M sucrose, 0·01 M ethylenediaminetetraacetate, pH 7·4, and one-half suspended in this medium and the other half suspended in water. Allowing for contaminating supernatant in the sediment from the centrifuging, the tonicity of the medium in which the latter sarcosomes were suspended was equivalent to about 0·01 M sucrose.

Anaerobic experiments. Spectrophotometric Thunberg tubes used. Phosphate, 0·03 M; malonate, 0·01 M; fluoride, 0·04 M; ADP, 2×10^{-4} M with sarcosomes suspended in isolation medium, 10^{-3} M with sarcosomes suspended in water; AMP, same as ADP; glucose, 0·02 M; ethylenediaminetetraacetate, 0·001 M; Mg, 0·005 M; αKg, 4×10^{-4} M; sarcosomes, 0·35 mg. protein/ml. (suspended in medium) or 0·40 (sarcosomes suspended in water); hexokinase, 128 and 113 units/mg. sarcosomal protein respectively. Method II used to measure phosphorylation with sarcosomes suspended in isotonic medium, Method I with sarcosomes suspended in water.

Aerobic experiments. α-Ketoglutarate as substrate: same as anaerobic expt., except ADP = AMP, 2×10^{-4} M with both preparations; αKg, 0·005 M; cytochrome *c*, $3{\cdot}4 \times 10^{-5}$ M. 30 min., 25°. Method II. Ascorbate as substrate: phosphate, 0·03 M; cytochrome *c*, $5{\cdot}2 \times 10^{-5}$ M; glucose, 0·02 M; ethylenediaminetetraacetate, 0·001 M; fluoride, 0·04 M; ADP, 10^{-3} M; AMP, 10^{-3} M; Mg, 0·005 M; ascorbate, 0·01 M; sarcosomes, 0·175 mg. protein/ml. (suspended in medium) or 0·198 mg. protein/ml. (suspended in water); hexokinase, 256 or 226 units/mg. sarcosomal protein respectively. 30 min., 25°. Method I.

	Sarcosomes suspended in: Isolation medium	Water
α-Ketoglutarate→ferricytochrome *c*		
Rate of reduction*	0·046	0·130
Ferricytochrome *c* (μatoms O equiv.)	0·314	0·314
$-\Delta\alpha$Kg (μmoles)	0·33	0·31
Δ esterified P (μatoms)	0·57	0·56
P:O	1·82	1·77
P:αKg	1·72	1·81
α-Ketoglutarate→O_2		
Q_{O_2} (μl. O_2/hr./mg. sarcosomal protein)	86	97
$-\Delta O$ (μatoms)	1·36	1·73
Δ esterified P (μatoms)	3·81	4·85
P:O	2·80	2·80
Ascorbate→O_2		
ΔO† (μatoms)	6·0	9·9
Δ esterified P (μmoles)	0·67	3·81
P:O	0·112	0·376

* Initial decrease of optical density at 580 mμ./min./mg. sarcosomal protein.

† Corrected for O_2 uptake in absence of sarcosomes.

Table 10. *Effect of 2:4-dinitrophenol on oxidative phosphorylation with α-ketoglutarate as hydrogen donor and either ferricytochrome c or oxygen as hydrogen acceptor*

Rat-heart sarcosomes (isolated in 0·28 M sucrose, 0·01 M ethylenediaminetetraacetate, pH 7·4), 0·54 mg. protein/ml.; phosphate, pH 7·4, 0·03 M; malonate, 0·01 M; fluoride, 0·04 M; AMP, 2×10^{-4} M; ADP, 2×10^{-4} M; αKg, 3×10^{-4} M; glucose, 0·02 M; ethylenediaminetetraacetate, 0·001 M; Mg, 0·005 M; hexokinase, 148 units/mg. sarcosomal protein. Dinitrophenol, 10^{-4} M, where indicated. Expts. with ferricytochrome c as hydrogen acceptor carried out in spectrophotometric Thunberg tubes.

		Oxygen as H-acceptor		Ferricytochrome *c* as H-acceptor	
		Without DNP	With DNP	Without DNP	With DNP
$-\Delta O$		2·15	?	0·464	0·464
αKg:	initial	4·81	4·81	0·94	0·94
	final	2·81	3·13	0·50	0·49
	Δ	−2·00	−1·68	−0·44	−0·45
~P:	initial	0·19	0·19	0·58	0·58
	final	0·20	0·15	0·44	0·43
	Δ	0·01	−0·04	−0·14	−0·15
HMP:	initial	0·01	0·01	0·02	0·02
	final	5·84	1·13	0·92	0·45
	Δ	5·83	1·12	0·90	0·43
ΔHMP + Δ~P		5·84	1·08	0·76	0·28
P:O		2·72	—	1·63	0·60
P:αKg		2·92	0·64	1·72	0·62

Table 11. *Comparison of phosphorylation ratios with α-ketoglutarate as hydrogen donor and either oxygen or ferricytochrome c as hydrogen acceptor*

Phosphate, 0·03 M; malonate, 0·01 M; fluoride, 0·04 M; Mg, 0·005 M; AMP, 2×10^{-4} M (except Expt. 1, $4{\cdot}8 \times 10^{-4}$ M and one tube of Expt. 8, 10^{-3} M); ADP, same as AMP; αKg, $3 \times 10^{-4} - 10^{-3}$ M; glucose, 0·01–0·03 M; ethylenediaminetetraacetate, 0·001–0·0017 M (except Expt. 1, zero); sarcosomes, 0·39–0·84 mg. protein/ml. (except Expt. 1, 1·3 mg./ml.); hexokinase, 127–181 units/mg. sarcosomal protein. 20–25°. Sarcosomes isolated from rat heart in 0·21–0·25 M sucrose, 0·01 M ethylenediaminetetraacetate, pH 7·4 except in Expt. 1 when pig heart and phosphate–saline were used. Apparatus A, side-arm Thunberg; B, spectrophotometric Thunberg.

			Ferricytochrome *c* as H-acceptor					
Expt.	O_2 as H-acceptor P:O	Apparatus	Time (min.)	$-\Delta O$* (μatom)	$-\Delta\alpha Kg$ (μmole)	Δ esterified P (μmole)	P:O	P:αKg
1	2·33†	A	4	0·300	0·27	0·54	1·75	1·94
2‡	2·60	A	12	0·456	0·44	0·88	1·94	2·00
3	2·63	B	11	0·505	0·46	0·86	1·69	1·87
4	2·71	B	13	0·464	0·44	0·76	1·63	1·72
5	2·71	B	14	0·469	0·45	0·78	1·67	1·74
6	2·64	B	14	0·489	0·46	0·73	1·50	1·59
7	2·70	B	13	0·464	0·42	0·79	1·70	1·89
8‡	2·80	B	12–16	0·314	0·32	0·56	1·79	1·77
9	2·80	B	18	0·314	0·33	0·57	1·82	1·72
						Means	1·72	1·80

* Oxygen equivalent of ferricytochrome *c*.
† Low value may be due to absence of ethylenediaminetetraacetate from reaction mixture.
‡ Mean values of two separate tubes.

such a way that the P:O ratios were low in the aerobic experiments, namely 1·77 and 1·50. The mean P:O ratios in the anaerobic experiments were 0·99 and 1·18 respectively.

Normal preparations, which in our hands give a phosphorylation ratio close to 3 in aerobic experiments (Slater & Holton, 1954), give a value just under 2 with ferricytochrome *c* as hydrogen acceptor.

DISCUSSION

It would be expected that some at least of the phosphorylation reactions in the respiratory chain would occur between substrate and ferricytochrome *c*. However, the only previous attempt to demonstrate this by direct experiment was unsuccessful (Friedkin & Lehninger, 1949; Lehninger, 1949). It is of some interest, therefore, that this phosphoryla-

tion has now been directly demonstrated. Moreover, the fact that the P:αKg ratio is much greater than unity and is sensitive to dinitrophenol shows that we are dealing not only with substrate-linked phosphorylation, but also with phosphorylation in the respiratory chain.

The demonstration of this phosphorylation was made possible by the development of a highly sensitive method of measuring oxidative phosphorylation (Slater, 1953*a*). This enabled the use of much smaller amounts of cytochrome *c* than Lehninger found necessary. This was important for two reasons: (i) even a single experiment with cytochrome *c* as hydrogen acceptor consumes large amounts of cytochrome *c* (0·5 μatom oxygen equivalent of cytochrome *c* represents 12·5 mg. protein, containing 0·45 % iron); (ii) the cytochrome *c* preparation might contain a small amount of an inhibitor. As already shown (Slater, 1950), a large amount of some preparations of cytochrome *c* ($1{\cdot}5 \times 10^{-4}$ M) made by the method of Keilin & Hartree (1945) does inhibit the phosphorylation slightly. However, even larger concentrations (6×10^{-4} M) of highly purified cytochrome *c* have no effect (Slater & Cleland, 1953).

Certain criticisms of the method employed to demonstrate phosphorylation as reported in the preliminary communication (Slater, 1950), which have been made by Lehninger (1951*b*) and by Lindberg & Ernster (1954) must be discussed. Lehninger (1951*b*) comments that 'the analytical methods employed...measure very small exchanges which approach, on a molar basis, the molar quantities of some of the electron transport factors involved. It is conceivable that such a complex pattern of exchange is "masked" with this type of measurement.' It is not clear exactly what electron transport factors Lehninger has in mind. Presumably, it is not cytochrome *c*, since in these experiments ferricytochrome *c* is used stoicheiometrically as one of the reactants, and acts as the ultimate acceptor of electrons, not an electron transport factor. Diphosphopyridine nucleotide (DPN), which is possibly an electron transport factor in the α-ketoglutaric oxidase system, is probably the respiratory catalyst in the highest concentration in the sarcosomes. Holton (1954*b*) has found that sarcosomes contain 0·006 μmole DPN/mg. sarcosomal protein, i.e. there was probably about 0·02 μmole DPN in the sarcosomes used in the experiment shown in Table 4. The reduction of this amount of DPN might be expected, at the most, to esterify 0·02 μatom phosphorus, which is only about 4 % of the phosphorus esterified by the reduction of the ferricytochrome *c*. In any case, it would be allowed for in the controls which included all the components of the reaction mixture, except the cytochrome *c*.

Lindberg & Ernster (1954), as well as repeating Lehninger's (1951*b*) criticism, comment:

> Slater, in his studies on the oxidation of α-ketoglutarate, employed considerable quantities of cytochrome *c* as electron acceptor, inhibiting the oxidation of cytochrome with cyanide, or with absence of oxygen. In these experiments a P:O ratio of between 0·98 and 2·20 was found. In the oxidation of α-ketoglutarate with oxygen in the same system the value varied between 1·67 and 2·79. The similarity between the two groups of values can scarcely be taken as conclusive evidence that no phosphorylation takes place between cytochrome *c* and oxygen.... It may be added here that the account of the experiments gives no direct evidence to exclude the possibility that a myokinase effect contributed to the formation of glucose 6-phosphate from the added ADP. Finally, it may be remarked that, as Hunter (1951) has suggested, cytochrome *c* may behave differently when it is introduced into the system and when it functions within the mitochondria.

The comparison between P:O ratios with oxygen and ferricytochrome *c* as hydrogen acceptors is useful only when the experiments are carried out under the same conditions. Reasons were given in the preliminary communication why it was considered that the valid comparison was between 2·5 with oxygen and 2·2 (which might be underestimated) with ferricytochrome *c*. The P:O ratio of 0·98 with ferricytochrome *c* was obtained in the presence of 0·01 M-HCN which inhibited the phosphorylation considerably. The comparison between values of 1·67–2·79 with oxygen and 0·98–2·20 with ferricytochrome *c* made by Lindberg & Ernster has no meaning and was not made in our previous paper.

Contrary to Lindberg & Ernster's statement, the published account of the preliminary experiments specifically states that the glucose 6-phosphate found was corrected for that attributable to the myokinase reaction. This has been previously discussed in detail (Slater, 1953*b*) and also in the present paper.

That added cytochrome *c* might behave differently from the endogenous cytochrome *c* of the particles has in fact been repeatedly stated in papers from this laboratory. Its relevance to the particular problem under investigation is difficult to test experimentally at the moment, but it would be somewhat surprising if there were more phosphorylation coupled with the reduction of added ferricytochrome *c* than of the endogenous cytochrome.

Although it has now been confirmed that some of the respiratory chain phosphorylation occurs before the reduction of ferricytochrome *c*, the conclusion that all the phosphorylation occurs in this part of the respiratory chain requires re-examination. The phosphorylation ratios found with ferricytochrome *c* as hydrogen acceptor in Table 11 are just under 2, compared with just over 2 in the previous paper.

Since the values in Table 11 were obtained under more strictly controlled conditions, they are to be preferred. The P:O ratio of 2·16 previously obtained in the anaerobic experiment may be slightly high because of residual oxygen, or because of a small analytical error. The two possible sources of error which could overestimate the yield of phosphorylation, namely residual oxygen or phosphorylation coupled with the dismutation reaction, have been eliminated in the present experiments.

Since the P:O ratio with oxygen is close to 3 and that with ferricytochrome *c* is just under 2, it is clear that the conclusion that the phosphorylation ratio with added cytochrome *c* is as high as with oxygen as hydrogen acceptor is not justified. However, the results now presented do not, of themselves, permit the conclusion that some of the phosphorylation in the respiratory chain occurs between cytochrome *c* and oxygen. Two explanations of the experiments are possible.

(1) Two steps in the respiratory chain between α-ketoglutarate and cytochrome *c* are associated with phosphorylation. One of these is presumably the substrate-linked phosphorylation, the other is some unidentified reaction, probably between DPNH and ferricytochrome *c*. (If, as many believe, the P:O ratio for the aerobic oxidation of α-ketoglutarate is 4, more than one reaction between DPNH and ferricytochrome *c* may be associated with phosphorylation.) There is an additional phosphorylation in the respiratory chain between cytochrome *c* and oxygen. This conclusion would agree with Nielsen & Lehninger's (1954) recent direct demonstration of phosphorylation with ferrocytochrome *c* as hydrogen donor.

(2) In the intact sarcosome, all the phosphorylation occurs between α-ketoglutarate and cytochrome *c* but when added cytochrome *c* is the hydrogen acceptor one of these phosphorylating steps is by-passed, thus

$$\rightarrow X \begin{cases} \nearrow \text{added cytochrome } c \\ \rightarrow \text{endogenous cytochrome } c \rightarrow \end{cases}$$

where the arrows show the direction of hydrogen (or electron) transfer and X is the immediate donor to the endogenous cytochrome *c*. It is possible that the reaction between X and endogenous cytochrome *c* is coupled with phosphorylation and that this reaction cannot be obtained with the added cytochrome *c*, perhaps because the cytochrome *c* becomes reduced during the course of the experiment. When oxygen is the hydrogen acceptor, the cytochrome *c* remains largely oxidized in the aerobic steady state.

While the first explanation is the easiest to reconcile with other work, the second should not be excluded, at least not until more is known about the mechanism of the phosphorylation obtained with ascorbic acid or ferrocytochrome *c*.

SUMMARY

1. Oxidative phosphorylation has been demonstrated with α-ketoglutarate as hydrogen donor and ferricytochrome *c* as acceptor. Since the ratio, phosphorus esterified:oxygen equivalent of ferricytochrome *c* reduced, was close to 2 and was sensitive to 2:4-dinitrophenol, respiratory chain phosphorylation must be involved.

2. These experiments were carried out under completely anaerobic conditions in order to prevent reoxidation of ferrocytochrome *c*. A sensitive enzymic method of detecting residual oxygen is described.

3. Respiratory inhibitors could not be used satisfactorily. Cyanide inhibits phosphorylation and combines with α-ketoglutarate; azide also inhibits phosphorylation and is a relatively ineffective respiratory inhibitor with intact sarcosomes. Sulphide rapidly reduces ferricytochrome *c*.

4. The combination between α-ketoglutarate and cyanide was shown to be a slow reaction.

5. The yield of oxidative phosphorylation with ferricytochrome *c* as acceptor was compared with that obtained with oxygen. The phosphorus:oxygen ratios with ferricytochrome *c* varied between 1·5 and 1·9 (mean 1·72) in nine experiments (phosphorus:α-ketoglutarate ratios, 1·6–2·0, mean 1·80), compared with just under three obtained with oxygen.

6. Two possible explanations of the results are suggested.

(i) Respiratory chain phosphorylation occurs between ferrocytochrome *c* and oxygen, as well as between substrate and ferricytochrome *c*.

(ii) All the phosphorylation occurs between substrate and the endogenous cytochrome *c* of the sarcosome, but the reaction with added ferricytochrome *c* by-passes a phosphorylating reaction.

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The Fractionation of Rabbit γ-Globulin by Partition Chromatography

By R. R. PORTER
National Institute for Medical Research, Mill Hill, London, N.W. 7

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Normal and immune rabbit γ-globulins have been examined by many techniques in attempts to relate the specific antigen-binding power of antibodies with some chemical or physical property, but no success has so far been achieved (see Porter, 1953). Thus, in the rabbit all antibodies are confined to the γ-globulin fraction and there is no difference in the electrophoretic behaviour of normal and immune γ-globulin (Tiselius & Kabat, 1939). In the ultracentrifuge they are indistinguishable, their molecular weight being about 170 000 (Kabat, 1939). Recent accurate amino acid analyses are now available for rabbit normal γ-globulin and antibodies to pneumococcal polysaccharide and they show no significant differences (Smith, McFadden, Stockell & Buettner-Janusch, 1955). The *N*-terminal pentapeptide sequence of rabbit antiovalbumin is the same as that of the inert γ-globulin with which it is associated (Porter, 1950), and more recently it has been found (McFadden & Smith, 1955) that antipneumococcal antibodies also have the same terminal pentapeptide sequence. It is therefore probable that, in the rabbit, the differences between antibody and inert γ-globulin, which are responsible for their specific binding power, are slight. It follows that any method not making use of the immunochemical specificity to separate antibody from inert γ-globulin would require a high resolving power. If successful, it might in turn give some indication as to the nature of the differences which must exist.

As the technique of protein chromatography with its different and perhaps higher resolving power became available it was of interest to see whether γ-globulin could be fractionated by this method and, if so, whether antibodies could be separated from inert protein.

In fact, using rabbit antiovalbumin, wholly inactive γ-globulin could be separated from the mixture but pure antibody (completely precipitable by antigen) could not be obtained. It was, however, found that the chromatographic pattern of the γ-globulin changed at different stages of immunization and, while one type of antibody was present in the earlier stages, this decreased and was replaced by a chromatographically distinct second type in the later stages.

EXPERIMENTAL

Materials

Celite 545 (a large-particle kieselguhr) was purchased from Johns-Manville Co. Ltd., Artillery Row, London. This was cleaned before use by stirring with 2N-HCl for 24 hr. It was

then filtered, washed with distilled water till free of acid, and dried at 105°. It was kept in a closed vessel over anhydrous $CaCl_2$.

Ethylene glycol monoethyl ether (ethyl cellosolve) and ethylene glycol monobutyl ether (butyl cellosolve) were distilled from $SnCl_2$ at about 1 cm. Hg pressure in an atmosphere of N_2. The redistilled solvents were kept only 1 or 2 weeks before use, and during that period were stored in dark bottles at 0°. In practice it was found essential to use relatively pure solvents as starting material, any containing more than a trace of peroxide and having an extinction coefficient in a 1 cm. cell greater than 0·4 at 280 mμ. were rejected. After distillation no peroxide could be detected and only fractions with an extinction coefficient not exceeding 0·05 were used.

Phosphate buffer was prepared by dissolving the required weight of KH_2PO_4 (A.R. grade) in water, adding KOH (A.R. grade) to give the required pH, measured with a glass electrode, and making to volume.

The ovalbumin used was a five-times recrystallized sample originally prepared in the laboratory of Professor R. K. Cannan.

Antibodies

The rabbit γ-globulin was prepared from fresh serum by Na_2SO_4 precipitation (Porter, 1950) using the method described by Kekwick (1940) for the preparation of human γ-globulin.

A suspension of alum-precipitated ovalbumin was used as antigen. It was prepared as follows: 10 ml. 4% (w/v) ovalbumin solution and 4·6 ml. M-$NaHCO_3$ were mixed in a centrifuge tube and 10 ml. 10% (w/v) potassium alum solution were dropped slowly in with rapid stirring. CO_2 was given off and a precipitate formed which was left overnight and then centrifuged. The precipitate was washed with 0·15 M-NaCl and then suspended in the same liquid to give a concentration of 16 mg. ovalbumin/ml. 1 part in 100000 merthiolate was added and the suspension stored at 2°.

Five rabbits were immunized as follows. Three injections by ear vein were given each week for 3 weeks: injection 1, 0·5 mg. ovalbumin; injection 2, 1 mg.; injection 3, 2 mg.; injection 4, 4 mg.; injections 5–9, 8 mg.

Approx. 20 ml. blood was taken from each rabbit 1 week after the last injection. The blood was allowed to clot and the serum pooled. The γ-globulin prepared by salt fractionation was dialysed free of Na_2SO_4 and freeze-dried. This is referred to as γ-globulin I.

Two months later four of the five rabbits were given six injections, twice weekly, of 8 mg. alum-precipitated ovalbumin. They were bled 1 week after the final injection, 40–50 ml. being taken from each rabbit. The γ-globulin from this blood is called γ-globulin II. Ten days later the rabbits were again bled without further injection. This preparation is γ-globulin III.

The antibody content of the three preparations was estimated as described by Kabat & Mayer (1948). This showed that: γ-globulin I contained 20% precipitable antibody; II, 33%; III, 30%.

Determination of protein

Estimation of protein concentration throughout this work was made by measuring the absorption of solutions at 280 mμ. in a 1 cm. cell in a Unicam spectrophotometer. In all cases the solutions were also read at 260 mμ. and if the absorption was due only to dissolved protein the ratio of the absorption at 260 mμ. to that at 280 mμ. was found to be 0·58. The double reading avoids the confusion which might arise from the absorption of non-protein impurities. The absorption at 280 mμ. was correlated with the weight of the protein and its nitrogen content. A neutral solution containing 1 mg. rabbit γ-globulin/ml. gave at 280 mμ. in a 1 cm. cell an extinction coefficient ($\log_{10} I_0/I$) of 1·3.

Chromatographic technique

Partition chromatography was used, the two liquid phases being produced by addition of strong phosphate buffer to an aqueous mixture of butyl and ethyl cellosolves. The phase diagrams from which this system was derived have been published and the factors determining choice of conditions for the satisfactory fractionation of a protein by partition chromatography have been discussed (Porter, 1954*a*). All the principal difficulties were met with γ-globulin; it is thrown out of solution by low concentrations of organic solvent and of salt, it is denatured by organic solvents at room temperature and also when adsorbed at an interface. It is easily adsorbed by solids such as kieselguhr, which are often used as support for the stationary phase in partition chromatography.

It followed therefore that all work would have to be carried out below 0° and that a very limited choice of suitable phase mixtures would be available. In fact, only one satisfactory system was found, and with this mixture chromatography was effective only in a very narrow range of concentration of the three components. Unfortunately the phase diagram of this system varied markedly with temperature, and while this gave a fourth variable available for adjustment in obtaining suitable conditions it meant that exact control (within 0·05°) was necessary.

It has previously been observed (Shepard & Tiselius, 1949) that serum globulins are adsorbed on to silica gel from neutral and acid solutions but that the adsorption decreases with increasing alkalinity and becomes small above pH 8·5. A similar effect was observed when the adsorption of rabbit γ-globulin was investigated. It was also found that Celite 545 had less adsorptive capacity than Hyflo Supercel, which in turn had less than that of silica gel. When adsorption occurred, it was always found to be partly irreversible. It was found best to use Celite 545 at pH 9·0, and in these circumstances there was no evidence of adsorption by the kieselguhr from either of the liquid phases used. The mixture, 480 g. water, 238 g. 2·5 M potassium phosphate buffer (pH 9·0) and 312 g. cellosolve (3 parts by vol. butyl cellosolve to 2 parts ethyl cellosolve) at $-3·1°$ was found to give a two-phase system in which the rabbit γ-globulin was distributed about 8:1 in favour of the aqueous phase. Hence the aqueous phase was the stationary phase and this was held by twice its weight of acid-washed Celite 545. More than 3 mg./ml. of γ-globulin was soluble in the organic solvent-rich phase. This mixture gave a single phase on cooling to $-5·0°$.

The preparation of the column and the details of the chromatography are the same as has been described for other proteins (Porter, 1954*a*) with the important exception that, because of the exacting nature of the system, precise control of all the variables is essential. Hence the phase mixture is prepared by weight rather than by volume. The sensitivity is such that with different batches of buffer slight

alteration of the mixture might be necessary to get the same spread of the γ-globulin peak.

Control of temperature was obtained by working in a cabinet whose air temperature was controlled at −3° by a contact thermometer. The cabinet was in a cold room at −7±2°. The column and the separating funnel which contained the phase mixture were jacketed and their temperature controlled by circulating liquid (4 parts water to 1 part ethylene glycol) from a bath at −3·1±0·05°. Fig. 1 shows diagrammatically the arrangement of the apparatus. It is important that the pump and stirrer should contribute very little heat to the bath. The outlet from the column must project into the lower compartment or the rapid air circulation caused by the fan evaporates the drops when forming.

Adequate time for equilibration of all glass apparatus in the cabinet must be allowed. The phase mixture is cooled to about −3°, poured into the jacketed separating funnel and stirred with a chain stirrer on a flexible drive for several hours to equilibrate at the correct temperature before the phases are allowed to settle out. When a column is poured it is allowed time to readjust to temperature, in case slight alteration has occurred in handling, before packing under a pressure of 5–10 cm. Hg. Mobile phase is run through till the blank reading of the eluate in the spectrophotometer at 280 mμ. is constant. The protein solution is allowed to sink into the column slowly. When the protein was dissolved in the mobile phase a trace of water was first added. Thus 0·04 ml. water would be added to 25 mg. globulin—sufficient to reduce it to a translucent paste—before adding 12 ml. of the moving phase. This greatly assists solution and does not appear to affect the chromatography. Most of the preparations used contained a little material which was insoluble in both the moving phase and saline. This slight denaturation is commonly observed when dialysing and freeze-drying γ-globulin.

When the precautions described have been taken, the same column with the same batch of phase mixture may be used a number of times with completely reproducible results and this offers the most satisfactory method of comparison of the different γ-globulin preparations.

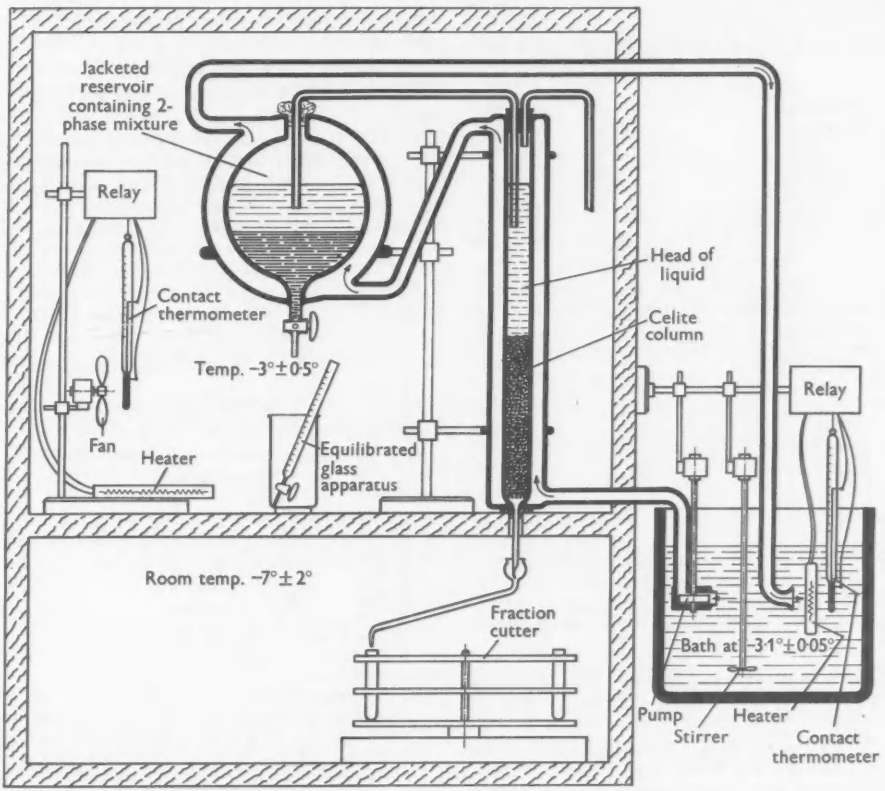

Fig. 1. Diagram of the apparatus used for chromatography with accurate temperature control at −3·1°. The cabinet containing the column, reservoir and glass apparatus is at −3°. The lower compartment containing the fraction cutter is at the room temperature of −7°±2°.

RESULTS

Normal γ-globulin

With γ-globulin prepared from the blood of stock rabbits, a chromatogram such as is shown in Fig. 2 was obtained. With a little more water in the phase mixture a sharp homogeneous peak with higher R value results (R as defined by Martin & Synge, 1941) and with less water present the peak becomes more and more spread without ever separating into discrete peaks. The recovery of soluble protein at the higher R value is more than 95 % but falls to 80 % or less as the peak becomes more and more spread. This suggests, as the average partition coefficient does not vary sharply in this range, that adsorption is contributing to the resolution of the protein.

Such an extended peak could arise from chromatography which was technically unsatisfactory but it would also occur if the protein was a mixture of a considerable number of very similar components. If the first alternative were correct, re-running any fraction on a second column would result in a chromatogram with the same peak R value and a similar spread. If the second explanation were correct, fractions from different parts of the original chromatogram would give a sharper zone and would have R values corresponding to that of the fraction from which they were taken.

The results of such an experiment are shown in Fig. 3 where fractions from a 25 g. column were put directly back on to a 5 g. column prepared with the same phase mixture. It is clear that although the protein was put back on the small column in a relatively much larger volume the different fractions appeared at the same R value as originally and were much less spread. This suggests that the chromatography is satisfactory, giving good resolution, and therefore that the original material is in fact a complex mixture of very similar components none of which are present in sufficient concentration to be isolated individually. The effective resolving power of the column is also shown by the localization of antibody found in the subsequent work with immune globulins.

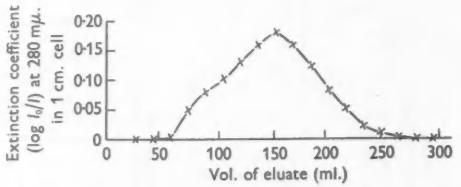

Fig. 2. Chromatogram of normal rabbit γ-globulin. Globulin (20 mg.) dissolved in moving phase (10 ml.) run on a column of 25 g. Celite 545 (diam. of column, 2·2 cm.), 15 ml. fractions. Points indicate end of collection.

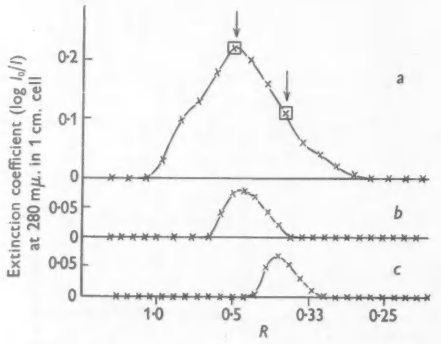

Fig. 3. (*a*) Chromatogram of normal rabbit γ-globulin. Conditions as in Fig. 2. (*b*) Portion of the peak fraction marked with arrow in (*a*) re-run on a 5 g. column (diam. 1 cm.), 2 ml. fractions. (*c*) Portion of slower-moving fraction marked in (*a*) re-run on the same 5 g. column (diam. 1 cm.), 2 ml. fractions.

Immune globulins

The immune globulins containing antiovalbumin were now run. 20 mg. normal γ-globulin were run on a 25 g. column and on the same column were run 20 mg. of each of the globulin preparations I, II and III. The chromatograms obtained are shown in Fig. 4. With the immune proteins, the tubes were combined as indicated to give 5 or 6 fractions. These

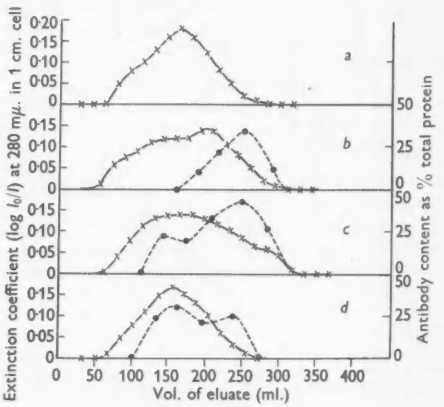

Fig. 4. Chromatograms of normal and immune γ-globulins. In each case 20 mg. globulin dissolved in 10 ml. moving phase was run on a 25 g. Celite 545 column. Column diameter, 2·2 cm. ×–×–× represents total protein concentration and •– – –• the antibody content expressed as percentage of the total protein in the fraction. *a*, normal γ-globulin; *b*, immune γ-globulin I; *c*, immune γ-globulin II; *d*, immune γ-globulin III. For description of the globulin preparations, see text.

were dialysed under 50–60 cm. Hg pressure against frequent changes of isotonic phosphate–saline (pH 7·4) until the volumes were reduced to about 5 ml. As there was insufficient material in each fraction to estimate antibody by adding increasing amount of ovalbumin to samples of the concentrated solution in the usual manner, the antibody in the whole of one fraction was precipitated in one tube. This was done by estimating the total protein content and adding sufficient ovalbumin to give optimal precipitation if 5% were antibody. If flocculation occurred, the supernatant was tested for excess antibody. If this was present, more antigen was added. By repetition all antibody was precipitated in the same tube, stood at 0° overnight and estimated as previously. Though tedious and probably less accurate than the standard procedure, it appeared to give satisfactory results.

It can be seen that the chromatographic pattern of the γ-globulin in preparations I and II is different from each other and from normal γ-globulin and γ-globulin III, which are essentially the same. The antibody, which is expressed as the percentage of the protein in each fraction, is associated with the slower-moving globulin in globulin I, but in addition it appears in the peak protein fractions in globulins II and III. There is a clear suggestion that there is a slower-moving type of antibody formed in the earlier stages of immunization and that this is replaced by a second, chromatographically distinct, type as immunization proceeds. The marked shift from γ-globulin II to III was not the result of further injections but only of heavy bleeding, which probably resulted in the release of much additional globulin into the blood. The antibody released was apparently of a different type from that previously present.

The chromatograms show that γ-globulin free of antibody can be obtained, but not antibody free of inert globulin.

DISCUSSION

Chromatography by adsorption and partition is being applied to proteins at an increasing rate (see Porter, 1954*b*). Published results have so far been largely confined to the smaller and more stable proteins, but the present extension to γ-globulin suggests that wider use may be possible. Susceptibility to denaturation following adsorption appears to be the greatest difficulty. Molecular size, as such, is unlikely to be important; it is probable that catalase (mol.wt. 240 000), with which the use of a three-component two-phase system for partitioning protein was first demonstrated (Herbert & Pinsent, 1948), could be chromatographed satisfactorily.

The heterogeneity of γ-globulins from all species has long been recognized. Dialysis resolves them into euglobulin and pseudoglobulin and antibodies against many different antigens may be present. Extensive electrophoretic investigation has been made and in bovine (Hess & Deutsch, 1949), equine (Deutsch & Nicol, 1948) and human (Alberty, 1949) γ-globulin, two fractions have been distinguished. During a study of fourteen proteins by reversible boundary spreading (Alberty, Anderson & Williams, 1948; Anderson & Alberty, 1948) none proved to be a single component, and normal and immune horse γ_2-globulin and human γ_1- and γ_2-globulin all showed clear evidence of heterogeneity. Though two distinct fractions were not apparent, two-way electrophoresis on paper again showed human γ-globulin to be a mixture of proteins (Kunkel & Tiselius, 1951). Rabbit γ-globulin has not been studied as fully as other species, but two electrophoretic fractions have not been reported nor has it been investigated by reversible boundary spreading. Possibly it is less heterogeneous than that of other species as the fluorodinitrobenzene technique shows only one terminal amino acid (Porter, 1950), while human, bovine (McFadden & Smith, 1953) and horse γ-globulin (Porter, unpublished) have a variety of terminal acids, none of which are present in molar proportions.

The results described here confirm the heterogeneity of rabbit γ-globulin and suggest that there are a large number of components whose chromatographic behaviour ranges about a mean value. Three possible explanations of this phenomenon are: (1) Different cells in the same or different tissues produce slightly varying types of γ-globulin. (2) The system responsible for γ-globulin synthesis is sensitive to changes in environmental conditions so that small differences in end product would result in response to such changes. (3) γ-Globulin has a labile structure such as proposed by Pauling (1940) and would be expected to show heterogeneous physical properties with the majority of molecules assuming the most stable configurations. An experimental approach to decide which, if any, of these explanations is the most probable should be possible.

The chromatography of the immune globulins shows that some inert globulin may be freed from antibody, but there seems no likelihood of complete separation by this method. That some differentiation can be achieved is in keeping with the results of Green & Anker (1954), who found, in the rabbit, that the relative rates of synthesis of antiovalbumin and normal γ-globulin were different. The interpretation of such work on rates of synthesis becomes more difficult if two distinct types of antibody are being produced against the same antigen, possibly at different rates, with the additional possibility that different fractions of γ-globulin may also be synthesized at different speeds.

The observation that one type of antiovalbumin is produced first and a different type later is reminiscent of the results found by Kekwick & Record (1941) in an electrophoretic study of horse serum during immunization with diphtheria toxoid. The antitoxin originally confined to the γ-globulin later appeared in a new β_2 or T fraction. Variation in distribution of antibodies with different characteristics, against different antigens has been observed in studies of rabbit sera by electrophoresis-convection (Cann, Campbell, Brown & Kirkwood, 1951) and of human sera by zone electrophoresis (Kuhns, 1954), but there was no evidence of progressive changes during immunization with the same antigen. The diversity of antibodies has been discussed by Kabat (1953). It remains to be investigated whether any immunological distinction, such as combining ratio, can be detected between the two chromatographically different types of antiovalbumin found in this work.

SUMMARY

1. Rabbit γ-globulin has been fractionated by partition chromatography using the two liquid phases produced by a mixture of potassium phosphate, water and ethyl and butyl cellosolves, with Celite 545 used to hold the stationary phase.

2. γ-Globulin from normal rabbits appeared to be a complex mixture of very similar components, none of which could be isolated individually.

3. Immune γ-globulin could be fractionated to give a partial separation of inert globulin from antibody.

4. One type of antiovalbumin appeared to be formed in the earlier stages of immunization, to be largely replaced by a chromatographically distinct second type as immunization continued.

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Studies in Detoxication

62. THE METABOLISM OF HALOGENOBENZENES. *ORTHO*- AND *PARA*-DICHLOROBENZENES*

BY W. M. AZOUZ, D. V. PARKE AND R. T. WILLIAMS
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

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Both *o*- and *p*-dichlorobenzenes are employed as insecticides. The *ortho* compound is used chiefly in soil against subterranean termites and to protect wood against beetles. The *para* compound is employed on a very large scale against the clothes moth and against insects infesting hides, furs and museum specimens, and also as a deodorant in block form (Shepard, 1951). *o*-Dichlorobenzene also has numerous other industrial uses, and a review of its toxic effects, which on the whole are relatively mild, has been given by Browning (1953). Little, however, is known about the metabolism of these

* Part 61, Robinson & Williams (1955).

compounds in animals. According to Callow & Hele (1926) the *ortho* isomer forms in dogs a mercapturic acid whose constitution was not determined. The *para* isomer, on the other hand, does not form a mercapturic acid (Baumann, 1883). More recently we have shown that *o*-dichlorobenzene yields 4:5-dichlorocatechol in the rabbit (Azouz, Parke & Williams, 1953), but we shall show in this paper that catechol formation is not a major path of metabolism of this compound. This is in contrast with monochlorobenzene, which yields 4-chlorocatechol as a major metabolite in rabbits (Smith, Spencer & Williams, 1950; Azouz *et al.* 1953).

EXPERIMENTAL

Materials and reference compounds

All melting points are corrected.

o-Dichlorobenzene, b.p. 178–179°, was purified according to Cohen & Hartley (1905). The following compounds were prepared by standard methods: 2:3-dichlorophenol, m.p. 57° (Holleman, 1917) and its *benzoate*, m.p. 81°, needle from ethanol (Found: C, 58·4; H, 3·2; Cl, 26·5. $C_{13}H_8O_2Cl_2$ requires C, 58·4; H, 3·0; Cl, 26·6%), and *toluene*-p-*sulphonate*, m.p. 95°, needles from methanol (Found: C, 49·0; H, 3·2. $C_{13}H_{10}O_2Cl_2S$ requires C, 49·2; H, 3·2%); 3:4-dichlorophenol, m.p. 71° (Holleman, 1917), and its *benzoate*, m.p. 97°, needles from ethanol (Found: C, 58·5; H, 3·0. $C_{13}H_8O_2Cl_2$ requires C, 58·4; H, 3·0%), and *toluene*-p-*sulphonate*, m.p. 111°, plates from ethanol (Found: C, 49·2; H, 3·2. $C_{13}H_{10}O_3Cl_2S$ requires C, 49·2; H, 3·2%); 4:5-dichlorocatechol, m.p. 105° (Peratoner, 1898); 2:3-dichloroquinol, m.p. 145° (Conant & Fieser, 1923).

3:4-Dichlorocatechol was synthesized as follows. 2:3-Dichlorophenol was converted into 3:4-*dichlorosalicylaldehyde* as described by Duff (1941) for the preparation of *o*-hydroxyaldehydes. The aldehyde, m.p. 94°, formed yellow needles from ethanol (yield 20%). (Found: C, 44·3; H, 2·0; Cl, 36·8. $C_7H_4O_2Cl_2$ requires C, 44·0; H, 2·1; Cl, 37·1%.) The 2:4-*dinitrophenylhydrazone* formed red needles from ethanol and yellow prisms from dioxan, both having m.p. 305–306° (decomp.). (Found: N, 15·6; Cl, 19·0. $C_{13}H_8O_5N_4Cl_2$ requires N, 15·1; Cl, 19·1%). The aldehyde (0·6 g.) in N-NaOH (5 ml.) was treated with 4 ml. of 20 vol. H_2O_2 (2 moles) (cf. Dakin, 1909.) The mixture darkened and became hot. After an hour, the cooled mixture was acidified with 2N-H_2SO_4 and then treated with a slight excess of saturated $NaHCO_3$ solution. The dichlorocatechol was extracted with ether, the ethereal solution dried over anhydrous Na_2SO_4 and then evaporated to dryness. The residue (0·5 g.) was recrystallized from light petroleum (b.p. 60–80°), whereby 3:4-*dichlorocatechol* was obtained as colourless needles, m.p. 99°. (Found: C, 40·1; H, 2·4; Cl, 39·7. $C_6H_4O_2Cl_2$ requires C, 40·3; H, 2·3; Cl, 39·6%.) It gave the typical catechol colour reactions with aqueous $FeCl_3$, being green in neutral and blue in $NaHCO_3$ solution. Its *ditoluene*-p-*sulphonate* formed colourless rhombs, m.p. 132°, from ethanol. (Found: Cl, 15·1. $C_{20}H_{16}O_6Cl_2S_2$ requires Cl, 14·6%.)

For the identification of the mercapturic acid of *o*-dichlorobenzene, 3:4-dichlorothiophenol and 3:4:3′:4′-tetrachlorodiphenyldisulphide were prepared. Synthetic 3:4-dichlorophenylmercapturic acid (0·2 g.) (Parke, 1955) in 2N-NaOH (5 ml.) was heated under reflux for 0·5 hr. The solution was acidified with 2N-H_2SO_4 and the thiophenol separated by steam distillation. 3:4-Dichlorothiophenol (0·1 g.) was obtained as a colourless solid, m.p. 28° after crystallization from ethanol. (Found: Cl, 39·9. $C_6H_4Cl_2S$ requires Cl, 39·6%.) The thiophenol (0·06 g.) in ethanol (5 ml.) was oxidized with ethanolic iodine solution and the mixture poured into water (10 ml.). The white precipitate of 3:4:3′:4′-*tetrachlorodiphenyldisulphide* was recrystallized from ethanol and formed colourless needles, m.p. 83–84°. (Found: C, 40·8; H, 1·9; Cl, 39·9. $C_{12}H_6Cl_4S_2$ requires C, 40·5; H, 1·7; Cl, 39·8%.)

p-Dichlorobenzene, m.p. 53°, was purified by recrystallization from ethanol. The following reference compounds were made: 2:5-dichlorophenol, m.p. 58° (Noelting & Kopp, 1905), and its benzoate, m.p. 68° (Holleman, 1917); 2:5-dichloroquinol, m.p. 168°, and its dibenzoate, m.p. 186° (Levy & Schultz, 1881).

Methods

Animals. Chinchilla rabbits, kept on a diet of 60 g. of rat cubes (diet 41; Associated London Flour Millers) and 100 ml. water per day, were used throughout this work. The dichlorobenzenes were administered by stomach tube, the *ortho* compound suspended in water and the *para* compound as 25% (w/v) solution in olive oil. Urine was collected daily.

Analytical methods. The urine was analysed daily for glucuronic acid by the modified naphthoresorcinol method of Paul (1951), ethereal sulphate by the turbidimetric method of Sperber (1948), total catechols according to Azouz *et al.* (1953) and mercapturic acid by the iodine titration method of Stekol (1936).

Determination of 2:3- *and* 3:4-*dichlorophenols.* 2:3-Dichlorophenol gives a red colour ($\lambda_{max.}$ 500 mμ.) when coupled with Brentamine Fast Red B salt (I.C.I. Ltd.; diazo salt from 5-nitro-*o*-anisidine with naphthalene 1:5-disulphonic acid) in alkaline solution. 3:4-Dichlorophenol does not give a red colour with this reagent, but could be estimated spectrophotometrically by a determination of the difference between its acid and alkaline spectra at 244 mμ., a correction being made for any 2:3-dichlorophenol present. The spectra of these phenols are shown in Fig. 1.

The urines from rabbits fed with *o*-dichlorobenzene were collected daily and each diluted to 200 ml. with water. The diluted urine (10 ml.) mixed with 5 ml. conc. HCl was heated under reflux for 3 hr. and the mixture then steam distilled, 100 ml. of distillate being collected. To 20 ml. of the distillate, containing <1 mg. of 2:3-dichlorophenol, 2 ml. of a freshly prepared aqueous solution of Brentamine Fast Red B salt (1 mg./ml.) were added, followed by 2 ml. of 2N-NH_4OH. The solution was mixed, diluted to 25 ml. with water and the colour density measured at 500 mμ. in a Unicam Spectrophotometer SP. 500. A blank was prepared by substituting 20 ml. water for the steam distillate. The standard curve for 0·1–1·0 mg. of the phenol in 25 ml. solution was a straight line. This procedure determined the 2:3-dichlorophenol present. The 3:4-dichlorophenol was estimated by diluting two 5 ml. portions of the distillate to 25 ml. with 0·1N-HCl and 0·1N-NaOH respectively, measuring the difference in absorption of the two solutions at 244 mμ. and then correcting for the absorption at 244 mμ. due to 2:3-dichlorophenol (ε at 244 mμ. being 8250 in 0·1N-NaOH and 200 in 0·1N-HCl, see Fig. 1). Blank estimations for 2:3- and 3:4-dichlorophenol were carried out on normal rabbit urines for several days before dosing

with *o*-dichlorobenzene. Using this method, the recovery, for example of 5 mg. of 2:3- and 25 mg. of 3:4-dichlorophenol added simultaneously to 20 ml. rabbit urine, was about 104±5% and 92±5% respectively.

Determination of 2:5-dichlorophenol. This steam-volatile phenol shows a maximum absorption at 241 mμ. ($\epsilon_{max.}$ 8500) in 0·1 N-NaOH. In 0·1 N-HCl the ε value at 241 mμ. is 500 (see Fig. 2 for spectra). The urines of rabbits which had received *p*-dichlorobenzene were collected daily and diluted to 200 ml. with water. The diluted urine (10 ml.) was mixed with 5 ml. conc. HCl, heated under reflux for 3 hr. and then steam distilled, 100 ml. of distillate being collected. Two 5 ml. portions of the steam distillate were then diluted to 25 ml. with 0·1 N-NaOH and 0·1 N-HCl respectively and the extinction at 241 mμ. was measured. The amount of 2:5-dichlorophenol excreted per day was then calculated from the expression

$$\text{mg. phenol/day} = 203{\cdot}75\ (E_{alk.} - E_{acid}),$$

where $E_{alk.}$ and E_{acid} are the observed extinctions in NaOH and HCl respectively and the factor 203·75 includes the dilutions, the mol.wt. of the phenol and the ε values in acid and alkali. Analyses were carried out on normal rabbit urine for 4 days before administering the *p*-dichlorobenzene, and normal urines contained the equivalent of 6–11 mg. dichlorophenol/day. The recovery of 100 mg. of 2:5-dichlorophenol added to 100 ml. rabbit urine was 93±5%.

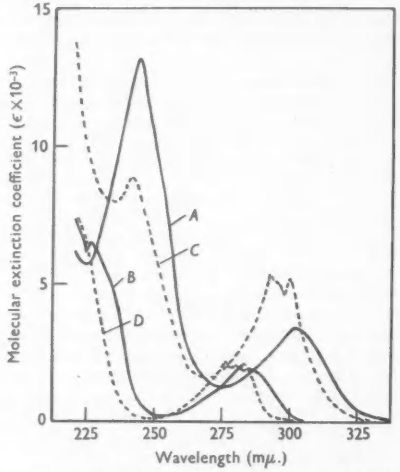

Fig. 1. The absorption spectra of 2:3- and 3:4-dichlorophenols. *A*, 3:4-Dichlorophenol in 0·1 N-NaOH ($\lambda_{max.}$ 244, 302 mμ.; $\epsilon_{max.}$ 13 250, 3250); *B*, 3:4-dichlorophenol in 0·1 N-HCl ($\lambda_{max.}$ 226, 282, 285 mμ.; $\epsilon_{max.}$ 6500, 1950, 1900); *C*, 2:3-dichlorophenol in 0·1 N-NaOH ($\lambda_{max.}$ 241, 292, 300 mμ.; $\epsilon_{max.}$ 8800, 5250, 5000); *D*, 2:3-dichlorophenol in 0·1 N-HCl ($\lambda_{max.}$ 277, 280, 283 mμ.; $\epsilon_{max.}$ 2150, 2000, 2050).

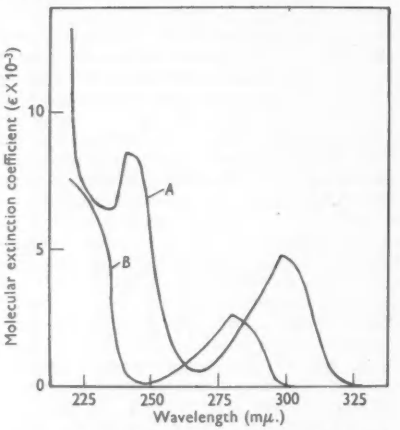

Fig. 2. The absorption spectra of 2:5-dichlorophenol. *A*, in 0·1 N-NaOH ($\lambda_{max.}$ 241, 298 mμ.; $\epsilon_{max.}$ 8500, 4800) and *B* in 0·1 N-HCl ($\lambda_{max.}$ 280 mμ., $\epsilon_{max.}$ 2600).

Chromatography

o-Dichlorobenzene metabolites. The possible phenolic metabolites of *o*-dichlorobenzene are 2:3- and 3:4-dichlorophenol, 3:4- and 4:5-dichlorocatechol and 2:3-dichloroquinol. The monophenols could be separated from the polyphenols by steam distillation, and therefore it was not necessary to separate all five phenols on the same chromatogram. Two solvent systems were used: *A*, benzene–acetic acid–water (1:1:2, v/v) and *B*, the *n*-butanol–ethanol–ammonia–ammonium carbonate buffer system of Fewster & Hall (1951). The two monophenols were not separated satisfactorily in these solvents, but the 2:3-dichlorophenol could be detected by colour reactions (see Tables 1 and 2). The three polyphenols were readily separated in both systems and detected by the colour reactions given in Table 2. The convenient amounts of the phenols used for the chromatograms were of the order of 50 μg.

Two mercapturic acids, namely 2:3- and 3:4-dichlorophenylmercapturic acids, are also possible metabolites. The pure compounds could be separated in the solvent system *B* (see Table 1). They were detected by spraying the paper with the buffered methyl red–bromophenol blue indicator of Fewster & Hall (1951) and they showed up as pink spots on a grey background. Proof of the nature of the mercapturic acid was obtained by other means (see below).

Table 1. *R_F values of dichlorophenols and dichlorophenylmercapturic acids*

Descending chromatography on Whatman no. 1 paper. In solvent *A* (benzene–acetic acid–water, 1:1:2, v/v) R_F values are after 6 hr. and in *B* (ethanol–*n*-butanol–3 N ammonia–ammonium carbonate buffer, 11:40:19, v/v; Fewster & Hall, 1951) 16 hr.

Compound	R_F values in solvent *A*	*B*
2:3-Dichlorophenol	—	0·84
3:4-Dichlorophenol	—	0·95
3:4-Dichlorocatechol	0·54	0·62
4:5-Dichlorocatechol	0·42	0·52
2:3-Dichloroquinol	0·38	—
2:5-Dichlorophenol	0·93	—
2:5-Dichloroquinol	0·33	—
2:3-Dichlorophenylmercapturic acid	—	0·70
3:4-Dichlorophenylmercapturic acid	—	0·75

Table 2. *Colour reactions of certain chlorinated phenols*

Phenols, 10–100 μg., spotted on Whatman no. 1 filter paper and the paper sprayed with the solutions described below. Figures in parentheses are pH values.

Compound	Gibb's reagent*	Brentamine Fast Red B† salt	Ferric chloride‡	Diazotized *p*-nitraniline§	Ammoniacal $AgNO_3$
2:3-Dichlorophenol	Blue-green (8–14)	Red	—	Orange	—
3:4-Dichlorophenol	None (<12) blue-green slowly (14)	None	—	Orange	—
3:4-Dichlorocatechol	Blue (9)	None	Green, neutral blue, $NaHCO_3$	—	—
4:5-Dichlorocatechol	Blue (9)	None	Green, neutral violet, $NaHCO_3$	—	Slight brown
2:3-Dichloroquinol	Blue (9)	None	Brown	Green→yellow	Brown
2:5-Dichlorophenol	Blue-green (8–14)	Red	—	Red	—
2:5-Dichloroquinol	Violet (8)	—	Brown	Orange-brown	Brown

* 2% ethanolic solution of 2:6-dichloroquinonechloroimide followed by saturated $NaHCO_3$ for pH 8; saturated borax solution for pH 9; Na_2CO_3 for pH>10.

† 0·1% aqueous solution of Brentamine Fast Red B salt followed by 2N ammonia.

‡ 1% aqueous $FeCl_3$, followed by saturated $NaHCO_3$.

§ Diazotized *p*-nitraniline followed by 2N-Na_2CO_3 (cf. Bray, Thorpe & White, 1950).

p-Dichlorobenzene metabolites. Preliminary tests had shown that mercapturic acids and catechols were not metabolites. 2:5-Dichloro-phenol and -quinol were readily separated chromatographically (see Table 1).

Isolation of metabolites

o-*Dichlorobenzene*

2:3- *and* 3:4-*Dichlorophenols.* *o*-Dichlorobenzene (1·25 g.) was fed to each of two rabbits and a 48 hr. urine collected. The urine was refluxed for 3 hr. with 0·5 vol. of conc. HCl. After cooling, the urine was continuously extracted with ether for 8 hr. The extract was evaporated and the residue steam distilled. The steam-volatile phenols were extracted from the distillate with benzene, which on evaporation left 1·2 g. of crude phenols. On benzoylation, the phenols yielded 0·7 g. (35% of dose) of crystalline benzoate, which on recrystallization from ethanol gave 3:4-dichlorophenyl benzoate, m.p. 96° and mixed m.p. 97°. (Found: C, 58·8; H, 3·0%.)

The 2-day urine of four rabbits which had collectively received 6 g. of *o*-dichlorobenzene was hydrolysed and the phenols were collected by steam distillation as above. 3:4-Dichlorophenol is less volatile in steam than its 2:3- isomer, which distils first. The crude phenols were therefore steam distilled until the distillate gave only a weak colour for the 2:3- isomer with Brentamine Fast Red B salt. This steam distillation was repeated 10 times and the phenol finally extracted into ether. The phenol was transferred to 2N-NaOH (5 ml.) and the solution treated with toluene-*p*-sulphonyl chloride (0·2 g.) in acetone (5 ml.). On pouring the product into water, 2:3-dichlorophenyl toluene-*p*-sulphonate was obtained (20 mg.), m.p. and mixed m.p. 93° after repeated recrystallization from ethanol and finally methanol. (Found: C, 49·1; H, 3·1%.)

The catechol fraction. 4:5-Dichlorocatechol has already been proved by isolation to be a metabolite of *o*-dichlorobenzene (Azouz *et al.* 1953). Paper chromatography, however, showed that 3:4-dichlorocatechol, but not 2:3-dichloroquinol, was also present. The urine of three rabbits which had collectively received 4·5 g. of *o*-dichlorobenzene was collected for 6 days. One-third of the urine was hydrolysed by heating for 3 hr. under reflux with 0·5 vol. of conc. HCl. The liberated phenols were separated by continuous extraction with ether for 8 hr. The extract was washed with water and the phenols transferred to 2N-NaOH. The alkaline solution was acidified and the monophenols were removed by steam distillation. The phenols not volatile in steam were now extracted from the residue into ether and then chromatographed in solvent systems *A* and *B* (see Table 1). Spraying with 2% aqueous $FeCl_3$ revealed two catechol spots, R_F 0·54 and 0·42 in the first solvent and 0·62 and 0·52 in the second. The faster spot corresponded to 3:4-dichlorocatechol and the other to the 4:5- isomer. From the size of the spots it was estimated that roughly 1% of the dose was appearing as 3:4- and 4% as 4:5-dichlorocatechol. (In the earlier publication (Azouz *et al.* 1953) it was reported that from *o*-dichlorobenzene urine, 4:5-dichlorocatechol ditoluene-*p*-sulphonate, m.p. 201° and an unidentified toluene-*p*-sulphonate of m.p. 98° were isolated. The latter ester has now been examined and appears to be a mixture of the esters of 3:4- and 2:3-dichlorophenol. On repeated recrystallization from ethanol it yielded pure 3:4-dichlorophenyl toluene-*p*-sulphonate, m.p. and mixed m.p. 111°.) Derivatives of 3:4-dichlorocatechol were not isolated in a pure state.

The mercapturic acid fraction. The urine (1900 ml.) of four rabbits which had collectively received 4 g. of *o*-dichlorobenzene was collected for 4 days. It was adjusted to pH 2 with 2N-H_2SO_4 and extracted continuously with ether for 16 hr. The extract and deposited gum were then extracted with saturated $NaHCO_3$, whereby phenols were left behind in the ether. The bicarbonate extract was then acidified with 2N-H_2SO_4 and the mercapturic acid transferred to chloroform (three extractions). Paper chromatography of this extract in solvent system *B* revealed one spot with R_F 0·75 corresponding to 3:4-dichlorophenylmercapturic acid (see Table 1). Attempts to crystallize the acid failed. The mercapturic acid fraction was therefore evaporated to dryness and the residue hydrolysed by refluxing for 0·5 hr. with 10 ml. 2N-NaOH. The solution was acidified with 2N-H_2SO_4 and the 3:4-dichlorothiophenol separated by steam distillation. The colourless crystalline thiophenol (60 mg. or 1·2% of the dose) was collected. It had m.p. 26° and did not

Table 3. *Quantitative excretion of metabolites of* o- *and* p-*dichlorobenzenes*

These results were obtained on a group of three rabbits studied simultaneously. Dose fed was 0·5 g./kg. body wt. Figures refer to percentage of dose excreted during 5–6 days after dosing; mean values are given in parentheses.

Compound fed	Glucuronide G	Ethereal sulphate E	Mercapturic acid M	Total conjugates (G+E+M)	Catechols	Dichlorophenols 2:3-	3:4-	2:5-
o-Dichlorobenzene	42	23	6·0	71	4·9	7·9	29	—
	51	15	3·2	69	2·2	9·8	29	—
	50	25	5·7	81	4·7	8·1	30	—
	(48)	(21)	(5·0)	(76)	(3·9)	(8·9)	(30)	—
p-Dichlorobenzene	32	30	0	62	0	—	—	35
	43	20	0	63	0	—	—	34
	34	30	0	64	0	—	—	36
	(36)	(27)	—	(64)	—	—	—	(35)*

* In addition to 2:5-dichlorophenol, about 6% of the dose of *p*-dichlorobenzene was excreted as 2:5-dichloroquinol.

depress the m.p. of the authentic compound. (Found: Cl, 39·5. Calc. for $C_6H_4Cl_2S$: Cl, 39·6%.) On oxidation of the thiophenol with ethanolic iodine solution and diluting with water, 3:4:3′:4′-tetrachlorodiphenyldisulphide was obtained as a colourless solid, m.p. and mixed m.p. 83°. (Found: Cl, 39·5. Calc. for $C_{12}H_6Cl_4S_2$: Cl, 39·8%.) These results show that *o*-dichlorobenzene is partly metabolized to 3:4-dichlorophenylmercapturic acid.

p-*Dichlorobenzene*

2:5-Dichlorophenol and 2:5-dichloroquinol. The urine of three rabbits, each of which had been given 1·5 g. of *p*-dichlorobenzene dissolved in olive oil, was collected for 6 days. A portion of the urine (equivalent to a dose of 4·1 g. *p*-dichlorobenzene) was heated under reflux with 0·3 vol. of conc. HCl for 3 hr. The urine was cooled and continuously extracted with ether for 8 hr. The phenols in the extract were transferred to N-NaOH, which was then acidified with 2N-H_2SO_4 and steam distilled. 2:5-Dichlorophenol, m.p. and mixed m.p. 56° was collected from the distillate by filtration (yield, 1·85 g. or 40% of dose). (Found: Cl, 43·1. Calc. for $C_6H_4OCl_2$: Cl, 43·5%.) 2:5-Dichlorophenyl benzoate was prepared and had m.p. and mixed m.p. 67°. (Found: Cl, 26·3. Calc. for $C_{13}H_8O_2Cl_2$: Cl, 26·6%.)

The aqueous residue after steam distillation was continuously extracted with ether for 8 hr. Evaporation of the ether extract left a dark brown residue. This was dissolved in 20 ml. 2N-NaOH and treated with 1 ml. benzoyl chloride. From the product there was isolated 2:5-dichloroquinol dibenzoate, m.p. and mixed m.p. 186° after crystallization from ethanol–benzene. (Found: Cl, 19·2. Calc. for $C_{20}H_{12}O_4Cl_2$: Cl, 18·3%.) The yield was 0·1 g. or 2·5% of the dose.

RESULTS

The quantitative results are summarized in Table 3. The major metabolites of both *o*- and *p*-dichlorobenzenes (70% for *o*- and 60% for *p*- in 6 days) are *O*-conjugates containing glucuronic and sulphuric acids. These conjugates reach a maximum on the first day after dosing with *o*-dichlorobenzene and on the second day with the *para* isomer, possibly owing to slower absorption of the latter. The *para* isomer, however, could not be detected in the faeces eliminated during the 6 days after dosing. The excretion of detectable metabolites appears to be complete with the *ortho* isomer in 6 days after dosing but with the *para* isomer the excretion of metabolites is still appreciable after this time.

The *O*-conjugates of *o*-dichlorobenzene yielded on acid hydrolysis 2:3- and 3:4-dichlorophenols and 3:4- and 4:5-dichlorocatechols. 3:4-Dichlorophenol is the major phenol (at least 30% of the dose) and reaches its peak excretion on the first day after dosing, whereas the 2:3-phenol (about 9% of the dose) reaches its peak on the second day (this was shown to occur in six different animals). Thereafter both phenols are excreted in approximately equal amounts. The catechols produced are minor metabolites (4% of the dose) and their peak excretion occurs on the first day after dosing and they cease to be measurable on the third day. Mercapturic acid excretion also reaches a peak on the first day after dosing and ceases to be measurable on the fifth day; the mercapturic acid is a minor metabolite (5% of the dose).

In the case of *p*-dichlorobenzene, only glucuronides and ethereal sulphates are excreted. There is no catechol or mercapturic acid excretion. Two phenols are produced namely 2:5-dichlorophenol which is the major one (35% of the dose) and 2:5-dichloroquinol which may account for about 6% of the dose (rough estimate from paper chromatograms). The glucuronide, ethereal sulphate and 2:5-dichlorophenol excretion reach their maximum on the second day after dosing.

The results in this paper are discussed, together with those of *m*-dichlorobenzene, in the succeeding paper (Parke & Williams, 1955).

SUMMARY

1. *o*-Dichlorobenzene, when fed to rabbits (0·5 g./kg.), is mainly oxidized to 3:4-dichlorophenol, which is excreted conjugated with glucuronic and sulphuric acids. Conjugates of 2:3-dichlorophenol, 4:5-dichlorocatechol and 3:4-dichlorocatechol are also excreted as minor metabolites.

2. *o*-Dichlorobenzene also gives rise to 3:4-dichlorophenylmercapturic acid as a minor metabolite (5% of the dose).

3. *p*-Dichlorobenzene (0·5 g./kg.) is mainly oxidized to 2:5-dichlorophenol which is excreted conjugated. 2:5-Dichloroquinol is also formed (about 6% of the dose) but in contrast with *o*-dichlorobenzene no mercapturic acid or dichlorocatechol is formed.

4. The excretion of the metabolites of *o*-dichlorobenzene is slow and appears to be complete in 5–6 days after dosing. With the *para* isomer, the excretion of metabolites is not complete and is still appreciable after 6 days.

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Studies in Detoxication

63. THE METABOLISM OF HALOGENOBENZENES. (*a*) *META*-DICHLOROBENZENE. (*b*) FURTHER OBSERVATIONS ON THE METABOLISM OF CHLOROBENZENE

BY D. V. PARKE AND R. T. WILLIAMS

Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

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Baumann (1883) concluded from experiments on dogs that *m*-dichlorobenzene did not form a mercapturic acid. Later experiments by Callow & Hele (1926), however, suggested the opposite and they concluded that in dogs *m*-dichlorobenzene formed about the same amount of mercapturic acid as monochlorobenzene. In the present work we shall show that in the rabbit *m*-dichlorobenzene does form a small amount of 2:4-dichlorophenylmercapturic acid, but the major metabolites are conjugates of 2:4-dichlorophenol. This phenol is a specific inhibitor of catalase (Goldacre & Galston, 1953).

EXPERIMENTAL

Materials and reference compounds

m-Dichlorobenzene (Light and Co.) was fractionally distilled. Three fractions were obtained: b.p. 169–171° (40% of the material), b.p. 171–172° (55%) and b.p. >172° (5%). The fraction, b.p. 171–172°, which was used in some experiments, was subsequently found to contain about 5% monochlorobenzene and will be referred to in the text as 'impure *m*-DCB'. Purified *m*-dichlorobenzene ('pure *m*-DCB') containing only traces of chlorobenzene was prepared as follows. Chlorobenzene is more readily sulphonated than *m*-dichlorobenzene. The 'impure *m*-DCB' (25 ml.) was heated on the water-bath with conc. H_2SO_4 (25 ml.) for 8 hr. with constant shaking (during this period equal volumes of chlorobenzene and conc. H_2SO_4 formed a single phase). The mixture was poured into water and the hydrocarbon layer, washed with water, dried over anhydrous Na_2SO_4 and distilled. The 'pure *m*-DCB' had b.p. 172–173°, d_4^{20} 1·288 and n_D^{22} 1·544 (literature values, b.p. 172°, d_4^{20} 1·288 and n_D^{21} 1·5457).

Reference compounds. The following compounds were either purchased or prepared by standard methods (melting points are corrected): 2:4-dichlorophenol (British Drug Houses Ltd.) m.p. 44° from ligroin, its benzoate, m.p. 97° (Mosso, 1887) and its toluene-*p*-sulphonate, m.p. 121° (Groves, Turner & Sharp, 1929); 3:5-dichlorophenol, m.p. 55° from ligroin was made from 3:5-dichloroaniline (Hodgson & Wignall (1927) give m.p. 68°), its benzoate had m.p. 66° from ethanol (Hodgson & Wignall (1926) give m.p. 55°) and its *toluene*-p-*sulphonate* had m.p. 104° (needles from ethanol) (Found: C, 49·2; H, 3·3; Cl, 23·1. $C_{13}H_{10}O_3Cl_2S$ requires C, 49·2; H, 3·2; Cl, 22·4%); 2:6-dichlorophenol, m.p. 65° (Holleman, 1917), was converted into its *toluene*-p-*sulphonate*, m.p. 72° (needles from ethanol), (Found: C, 49·5; H, 3·5; Cl, 22·1%); 3:5-dichlorocatechol, m.p. 84° (Dakin, 1909), was prepared from 2:4-dichlorophenol via 3:5-dichlorosalicylaldehyde (Duff, 1941) and converted into its *ditoluene*-p-*sulphonate*, m.p. 145° (short needles from

ethanol), (Found: Cl, 14·1. $C_{20}H_{16}O_6Cl_2S_2$ requires Cl, 14·4%); 2:6-dichloroquinol, m.p. 164°, was prepared according to Dakin (1909) from 3:5-dichloro-4-hydroxybenzaldehyde (Biltz, 1904), and its dibenzoate, m.p. 113°, according to Levy (1883) who gives m.p. 105°.

2:4-Dichlorothiophenol, m.p. 22° (Baddeley & Bennett (1933) give m.p. 20°) was prepared by alkaline hydrolysis of synthetic 2:4-dichlorophenylmercapturic acid (Parke, 1955) followed by steam distillation of the acidified hydrolysate. It was oxidized to 2:4:2′:4′-tetrachlorodiphenyldisulphide, m.p. 84° (Baddeley & Bennett, 1933), with ethanolic iodine solution.

Reference compounds related to monochlorobenzene. Chlorobenzene (British Drug Houses Ltd.), b.p. 131–132°, was redistilled. 4-Chlorocatechol, m.p. 91°, chromatographically free from catechol and 3-chlorocatechol (cf. Willstätter & Müller, 1911) was prepared from 5-chlorosalicylaldehyde according to the method of Dakin (1909); 3-chlorocatechol, m.p. 45° (Willstätter & Müller, 1911), 4-chlororesorcinol, m.p. 89° (British Drug Houses Ltd.) and chloroquinol, m.p. 103° (British Drug Houses Ltd.), were prepared or purchased, and purified.

2-Chlorothiophenol, b.p. 205°, 3-chlorothiophenol, b.p. 205°, and 4-chlorothiophenol, m.p. 54° (cf. Daccomo, 1891), were prepared by hydrolysis of the corresponding mercapturic acids (cf. Parke, 1955) with alkali followed by steam distillation after acidification. 4:4′-Dichlorodiphenyldisulphide, m.p. 71° (cf. Otto, 1868) was obtained by oxidation of 4-chlorothiophenol with ethanolic iodine.

Methods

Animals and general analytical methods are as described in the preceding paper (Azouz, Parke & Williams, 1955).

Determination of 2:4- and 3:5-dichlorophenols. 3:5-Dichlorophenol gives a red colour with Brentamine Fast Red B salt in the presence of ammonia, which is proportional to the amount of phenol, whereas 2:4-dichlorophenol gives no appreciable colour with this reagent. These phenols are the only chlorinated monophenols present in *m*-dichlorobenzene urine. They were therefore estimated together by the method used for 2:3- and 3:4-dichlorophenols described in the preceding paper (Azouz *et al.* 1955). The spectra in acid and alkali of 2:4- and 3:5-dichlorophenols are shown in Fig. 1 (ϵ at 244 mμ. for 2:4-dichlorophenol is 9500 in NaOH and 300 in HCl, for 3:5-dichlorophenol, 6600 in NaOH and 150 in HCl). When 2:4- and 3:5-dichlorophenols were added to normal urines their recovery by this method was satisfactory; thus, for example, when 250 mg. of 2:4-compound and 25 mg. of 3:5-compound were added to 200 ml. normal urine, the recovery was 98±5% and 93±5% of the respective compounds.

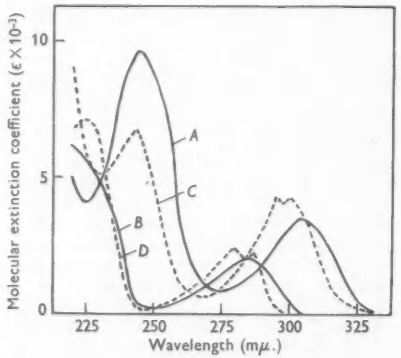


Fig. 1. The spectra of 2:4- and 3:5-dichlorophenols. *A*, 2:4-dichlorophenol in 0·1 N-NaOH ($\lambda_{max.}$ 244, 304 mμ.; $\epsilon_{max.}$ 9500, 3400) and *B* in 0·1 N-HCl ($\lambda_{max.}$ 285 mμ.; $\epsilon_{max.}$ 2000); *C*, 3:5-dichlorophenol in 0·1 N-NaOH ($\lambda_{max.}$ 243–244, 295, 300 mμ.; $\epsilon_{max.}$ 6600, 4100, 4200) and *D* in 0·1 N-NaOH ($\lambda_{max.}$ 223–224, 279, 287 mμ.; $\epsilon_{max.}$ 7000, 2300, 2100).

Chromatography

The possible phenolic metabolites of *m*-dichlorobenzene are 2:4-, 2:6- and 3:5-dichlorophenols, 3:5-dichlorocatechol and 2:6-dichloroquinol. The three monophenols can be separated from the polyphenols by steam distillation and then on paper in solvent system *B* (Table 1). They are also distinguishable by colour reactions (see Table 2). The polyphenols are readily separable in solvent system *A*.

The 2:4- and 3:5-dichlorophenylmercapturic acids are only just separable in solvent system *B* (see Table 1). The 2:6- isomer is readily distinguished from the other two. They were detected by the indicator reagents described by Fewster & Hall (1951). Better separation of these mercapturic acids was obtained after conversion into the corresponding thiophenols. The dichlorophenylmercapturic acids (50 mg.) were heated at 100° for 30 min. in 5 ml. N-NaOH. The solutions were acidified with 2 N-H_2SO_4 and steam distilled, and the volatile dichlorothiophenols extracted into ether. They were chromatographed on paper in about 50 μg. quantities in solvent system *B* and detected by spraying first with aqueous 0·01 N-I_2 containing 10% 2 N-H_2SO_4 and then with 2% starch solution. The thiophenols showed up as white spots on dark blue background (see Table 1).

The possible dihydric phenols of monochlorobenzene, 3- and 4-chlorocatechol, 4-chlororesorcinol and chloroquinol

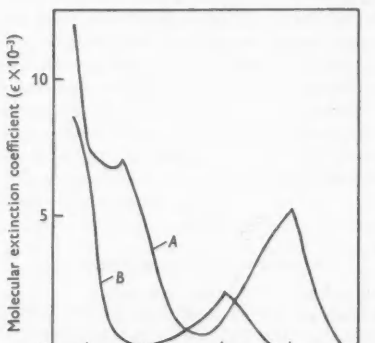


Fig. 2. The spectra of 2:6-dichlorophenol. *A* in 0·1 N-NaOH ($\lambda_{max.}$ 238, 301 mμ.; $\epsilon_{max.}$ 7100, 5250) and *B* in 0·1 N-HCl ($\lambda_{max.}$ 276 mμ.; $\epsilon_{max.}$ 2250).

Table 1. *R_F values of chlorophenols and mercapturic acids related to chlorobenzene and* m-*dichlorobenzene*

Descending chromatography on Whatman no. 1 paper. In solvent *A* (benzene–acetic acid–water, 1:1:2, v/v) values are after 6 hr. and in *B* (ethanol–*n*-butanol–3N ammonia–ammonium carbonate buffer (11:40:19, v/v); Fewster & Hall, 1951) after 16 hr. R_F values followed by *A* were determined in solvent *A*, the others in solvent *B*.

Compound	R_F values	
2:4-Dichlorophenol	0·85	0·75* (buff)
2:6-Dichlorophenol	0·73	0·77* (pale yellow)
3:5-Dichlorophenol	0·94	0·80* (buff)
4-Chlorocatechol	0·33 *A*	—
3-Chlorocatechol	0·41 *A*	—
4-Chlororesorcinol	0·19 *A*	—
Chloroquinol	0·17 *A*	—
3:5-Dichlorocatechol	0·51 *A*	—
2:6-Dichloroquinol	0·39 *A*	—
L-2-Chlorophenylmercapturic acid	0·56	—
L-3-Chlorophenylmercapturic acid	0·61	—
L-4-Chlorophenylmercapturic acid	0·60	—
L-2:4-Dichlorophenylmercapturic acid	0·61	—
L-2:6-Dichlorophenylmercapturic acid	0·53	—
L-3:5-Dichlorophenylmercapturic acid	0·65	—
2-Chlorothiophenol	0·50	—
3-Chlorothiophenol	0·61	—
4-Chlorothiophenol	0·59	—
2:4-Dichlorothiophenol	0·02	—
2:6-Dichlorothiophenol	0·56	—
3:5-Dichlorothiophenol	0·39	—

* The phenols were first coupled with diazotized *p*-nitraniline and the product was chromatographed.

Table 2. *Colour reactions on paper of various chlorophenols*

The figures in parentheses are approximate pH values; — means not tested.

Phenol	Gibb's reagent*	Brentamine Fast Red B salt†	Diazotized sulphanilic acid‡	Diazotized *p*-nitraniline§	$FeCl_3$‖	Ammoniacal $AgNO_3$
2:4-Dichlorophenol	Pale blue (8–10), olive green (12)	Pale yellow	Orange-red slowly	—	—	—
2:6-Dichlorophenol	Pale green (8), deep green (10–12)	Red	Lemon-yellow	—	—	—
3:5-Dichlorophenol	Deep blue (8–10), blue (12)	Red	Orange-yellow	—	—	—
3-Chlorocatechol	No colour (7), blue→brown (9)	—	—	—	Green (7), blue (9)	Pale brown
4-Chlorocatechol	Blue (7), blue (9)	—	Red	Blue-violet	Green (7), violet (9)	Pale brown
3:5-Dichlorocatechol	Blue (>8)	—	—	—	Green (7), violet (9)	Pale brown
4-Chlororesorcinol	Violet (7), blue-violet (8)	—	Deep yellow-brown	Deep yellow-brown	Brown (7)	Pale brown
Chloroquinol	Pink (7), green (8)	—	Greyish pink	Pale yellow	Brown (7)	Brown
2:6-Dichloroquinol	Green (7–10)	—	—	—	—	Brown

* 2% ethanolic solution of 2:6-dichloroquinonechloroimide followed by saturated $NaHCO_3$ for pH 8; saturated borax solution for pH 9 and 2N-Na_2CO_3 for pH>10.
† 0·1% aqueous solution of Brentamine Fast Red B salt followed by 2N ammonia.
‡ Diazotized sulphanilic acid followed by 2N-Na_2CO_3.
§ Diazotized *p*-nitraniline followed by 2N-Na_2CO_3.
‖ 1% aqueous $FeCl_3$ followed by saturated $NaHCO_3$.

are separable on paper in solvent system *A* (see Table 1) and are detectable by their colour reactions (Table 2). The 2-, 3- and 4-chlorophenylmercapturic acids were not readily separated on paper, although the *ortho* could be distinguished from the *meta* and *para* isomers. No better separation could be achieved on conversion of the mercapturic acids into the thiophenols, except that the *ortho* is more readily distinguished from the *meta* and *para* isomers (see Table 1). The thiophenols were detected on the paper by spraying with iodine and starch as for the dichlorothiophenols, or by Folin & Ciocalteu's reagent (British Drug Houses Ltd.) followed by 2N-Na_2CO_3, which gives a blue spot with the thiophenols, or by spraying with a mixture of 20% (w/v) aqueous hydroxylamine hydrochloride and a solution of $CuCl_2$ (1·5 g.), NH_4Cl (3 g.) and ammonia (sp.gr. 0·88; 3 ml.) in 50 ml. water (Feigl, 1954), whereby the thiophenols show up as yellow-brown spots on a white background.

Isolation and detection of metabolites

Phenols. A rabbit was fed 1·5 g. 'pure *m*-DCB' and the urine collected during 4 days. The urine was made 3N with respect to HCl and heated under reflux for 3 hr. After cooling, it was continuously extracted with ether for 8 hr. The extract was washed with 2N-$NaHCO_3$ and the washings were discarded and then the phenols transferred to 2N-NaOH. After acidifying with 2N-H_2SO_4, the monophenols were separated from the solution by steam distillation and taken up in ether, and the dihydric phenols extracted from the residue with ether.

The monophenol extract gave a deep blue colour with Gibb's reagent at pH 8–14 and a red colour with Brentamine Fast Red B salt. These tests indicated the presence of 3:5- or 2:6-dichlorophenol or both. Chromatography in solvent system *B* revealed two spots of R_F 0·85 and 0·93 not present in normal rabbit urine. Spraying with diazotized sulphanilic acid and Brentamine Fast Red B salt, showed the spot of R_F 0·85 to be 2:4-dichlorophenol and the one with R_F 0·93 to be 3:5-dichlorophenol. The 2:6- isomer was not present. The presence of these two phenols was confirmed by coupling the extract with diazotized *p*-nitraniline before chromatography. Chromatography in solvent system *B* gave two buff spots with R_F 0·75 (2:4- compound) and 0·81 (3:5-compound) (cf. Table 1).

Chromatography of the dihydric phenol fraction in solvent system *A* revealed two spots ($FeCl_3$ spray), one of R_F 0·51 corresponding to 3:5-dichlorocatechol and a weak one of R_F 0·33 corresponding to 4-chlorocatechol. When 'impure *m*-DCB' was used, the spot corresponding to 4-chlorocatechol was much more intense. Chromatography in this and other systems failed to reveal the presence of any 2:6-dichloroquinol.

Isolation of 2:4-dichlorophenol. The urine from three rabbits which had received 1·5 g. each of 'impure *m*-DCB' was collected for 4 days, made 3N with respect to HCl and heated under reflux for 3 hr. The phenols were extracted with ether as above. The monophenol fraction was separated by steam distillation. The 3:5-dichlorophenol, as indicated by the red colour given by the distillate with Brentamine Fast Red B salt, appeared in the first fractions, but we failed to obtain a derivative of it. The phenols in the distillate were converted into toluene-*p*-sulphonates (1·72 g. or 19% of the dose) and recrystallization from ethanol yielded 2:4-dichlorophenyl toluene-*p*-sulphonate, m.p. and mixed m.p. 119°. (Found: Cl, 22·7. Calc. for $C_{13}H_{10}O_3Cl_2S$: Cl, 22·4%.) In a second experiment 1·45 g. (25% of the dose of 3 g.) of 2:4-dichlorophenyl toluene-*p*-sulphonate were obtained.

Working up the monophenol fraction chromatographically using powdered cellulose did concentrate the 3:5-isomer, but no pure derivative of it could be isolated.

3:5-Dichlorocatechol. The phenol fraction not volatile in steam was chromatographed on cellulose powder (Whatman, standard grade) in solvent system *A*. Two catechol bands were formed. These were eluted with the same solvent and the catechols in each transferred to a little 2N-NaOH. Each fraction was then treated with toluene-*p*-sulphonyl chloride and the sulphonates formed isolated in the usual manner. From the first band a small quantity of crystalline material was isolated which on repeated crystallization from ethanol and ethanol–ethyl acetate mixtures yielded a few crystals of 3:5-dichlorocatechol ditoluene-*p*-sulphonate, m.p. and mixed m.p. 143°. The second fraction which presumably contained 4-chlorocatechol yielded no crystalline material.

The mercapturic acid fraction. The urine of rabbits fed with 0·5 g./kg. of 'pure *m*-DCB' (total 3 g.) was collected for 6 days and adjusted to pH 2 with 2N-H_2SO_4. It was then continuously extracted with ether for 8 hr. The extract was washed with water and the carboxylic acids present transferred to saturated $NaHCO_3$. The bicarbonate extract was washed twice with ether to remove any contaminating phenols and then acidified and the free acids were transferred to ether. The ether extract was chromatographed using solvent system *B* (Table 1) and acid spots were detected with the buffered methyl red–bromophenol blue indicator of Fewster & Hall (1951). The extract gave one spot of R_F 0·61. No crystalline mercapturic acid was isolated, but the fraction, on alkaline hydrolysis, acidification and steam distillation, yielded a thiophenol (30 mg. or 8% of dose) m.p. 15–20° (2:4-dichlorothiophenol has m.p. 22°) and R_F 0·03 in solvent system *B* (see Table 1). On oxidation with ethanolic iodine, 2:4:2′:4′-tetrachlorodiphenyldisulphide, m.p. 75° was obtained. On recrystallization the m.p. was raised to 84°, not depressing the m.p. of the authentic disulphide, m.p. 84°. (Found: Cl, 39·2. Calc. for $C_{12}H_6Cl_4S_2$: Cl, 39·8%.) These results show that the mercapturic acid is 2:4-dichlorophenylmercapturic acid.

Experiments with monochlorobenzene

The dihydric phenol fraction. Three rabbits were fed 1·5 g. each of chlorobenzene and the urine was collected for 5 days. One-half of the urine was hydrolysed by refluxing for 3 hr. with 0·5 vol. of concentrated HCl. After cooling, the urine was extracted with ether continuously for 8 hr. The phenols in the extract were transferred to 2N-NaOH. The alkaline extract was acidified and monophenols removed by steam distillation. The non-volatile phenols in the residue were then extracted with ether. This ether extract was then used for paper chromatography in solvent system *A* (see Table 1). One catechol only was detected of R_F 0·33, which corresponds to 4-chlorocatechol. No other dihydric phenol was demonstrable in amounts greater than 0·2% of the dose (the limit of detection).

The mercapturic acid fraction. The other half of the above urine was acidified to pH 2 with 2N-H_2SO_4 and continuously extracted with ether for 16 hr. Carboxylic acids in the extract were extracted into 2N-Na_2CO_3 and this was re-extracted with ether to remove any phenols. The solution was then acidified and the carboxylic acids were transferred

to ether. Paper chromatography of this ether extract in solvent system *B* (see Table 1) at 22° revealed one spot (R_F 0·59) detected by the buffered methyl red–bromophenol blue indicator mixture. This result did not permit us to decide whether *o*-, *m*- or *p*-chlorophenylmercapturic acid or all three were present.

o- and *m*-Chlorophenylmercapturic acids are more soluble in water than the *para* isomer (Parke, 1955), hence these isomers if they occurred in small amounts would be readily lost on recrystallization of the mercapturic acid fraction from chlorobenzene urine. However, on alkaline hydrolysis these acids are converted into the chlorothiophenols, which can be recovered almost quantitatively by steam distillation. The presence of small amounts of *o*- and *m*-chlorothiophenol in *p*-chlorothiophenol are readily detected, since the former are liquids at ordinary temperatures and affect the m.p. of the *para* isomer. For example, a mixture of 10 mg. *o*- and 100 mg. *p*-chlorophenylmercapturic acid was hydrolysed with 5 ml. N-NaOH for 0·5 hr. at 100°. After acidification and steam distillation the chlorothiophenol isolated had m.p. 45–50° (pure *para* isomer, m.p. 54°) and on oxidation gave a disulphide, m.p. 55–60° raised to 67–68° on recrystallization from ethanol (4:4′-dichlorodiphenyldisulphide has m.p. 71°).

The ether extract of mercapturic acids from chlorobenzene urine was evaporated to dryness and the residue hydrolysed with 10 ml. N-NaOH for 0·5 hr. at 100°. The hydrolysate was acidified and steam distilled. The solid product (0·4 g.) was filtered off and dried. It had m.p. 54° (without recrystallization) not depressing the m.p. of 4-chlorothiophenol, m.p. 54°. On oxidation with ethanolic iodine it yielded pure 4:4′-dichlorodiphenyldisulphide, m.p. and mixed m.p. 72° without recrystallization. Paper chromatography of the thiophenol fraction (see Table 1) yielded one spot of R_F 0·60, which excludes the *ortho* isomer (R_F 0·50). We conclude therefore that chlorobenzene urine contains no detectable *o*- or *m*-chlorophenylmercapturic acid.

RESULTS

The quantitative results are expressed in Table 3. Measurable metabolites of *m*-dichlorobenzene cease to be excreted in 5 days after dosing, but in this time not more than half the dose can be accounted for as total conjugates (Table 3). The major metabolites are glucuronides and ethereal sulphates which reach their peak excretion usually on the first day after dosing. On hydrolysis the urine yields 2:4- and 3:5-dichlorophenols and 3:5-dichlorocatechol. The first is the major phenol and accounts for at least 20 % of the dose. 3:5-Dichlorophenol is a relatively minor metabolite. 2:4-Dichlorophenylmercapturic acid and 3:5-dichlorocatechol are also minor metabolites and are excreted in greatest amount on the first day after dosing. Both catechol and mercapturic acid excretion continue until the fifth day.

DISCUSSION

The metabolites of *o*- and *m*-dichlorobenzenes are qualitatively similar but quantitatively completely different from that of monochlorobenzene (see Table 4). With the mono- compound, catechol and mercapturic acid are major metabolites, whereas with the *o*- and *m*-dichloro- compounds they are only formed in small amounts. *p*-Dichlorobenzene

Table 3. *Excretion of metabolites of* m-*dichlorobenzene*

Dose fed 0·5 g./kg. wt. of rabbit. Figures refer to percentage of dose excreted during 5 days after dosing. Figures in parentheses are mean values. Superior figures indicate the number of experiments.

	Glucuronide	Ethereal sulphate	Mercapturic acid	Total conjugates	Catechols	Dichlorophenols 2:4-	Dichlorophenols 3:5-
'pure *m*-DCB'	38	8	8·3	54	2·1	24	4·6
	30	7	10·6	48	2·9	20	3·0
	40	6	13·8	60	2·9	19	3·2
	(36)	(7)	(10·9)	(54)	(2·6)	(21)	(3·6)
'impure *m*-DCB'	30–37 (32)[6]	9–16 (12)[6]	8·8–8·9 (8·9)[3]	49–53 (51)[3]	3·7–4·7 (4·1)[5]	—	—

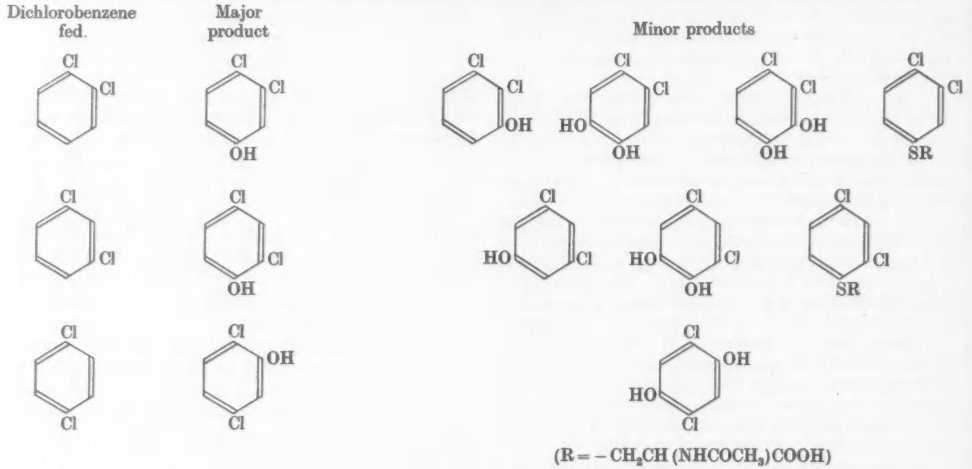
Table 4. *Excretion of metabolites of dichlorobenzenes by rabbits*

Dose fed was 0·5 g./kg. body wt. Results are expressed as percentage of fed dose.

Metabolite	Dichlorobenzene *ortho*	Dichlorobenzene *meta*	Dichlorobenzene *para*	Monochlorobenzene*
Glucuronide	48	36	36	25
Ethereal sulphate	21	7	27	27
Mercapturic acid	5	11	0	20
Total conjugates	74	51	63	72
Monophenols	39	25	35	3
Catechols	4	3	0	27
Quinols	0	0	0	—
Period of excretion	6† days	5† days	*ca.* 6 6‡ days	3 days

* From Azouz *et al.* (1952, 1953) and Spencer & Williams (1950).
† Excretion of metabolites apparently complete.
‡ Excretion of metabolites not complete in 6 days.

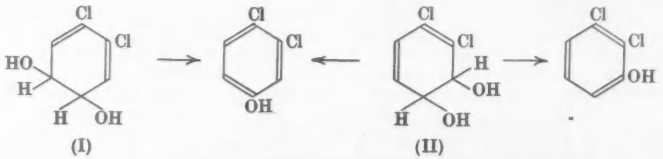
Table 5. *Orientation of the metabolites of the isomeric dichlorobenzenes excreted by the rabbit*



differs from all three since it forms neither a catechol nor a mercapturic acid. The orientations of the metabolites formed are summarized in Table 5.

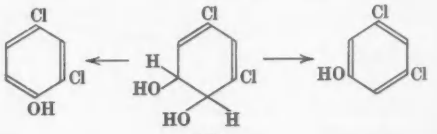
Phenols

The major metabolites of the dichlorobenzenes are monophenols. In the *ortho* and *meta* compounds, the hydroxyl groups in these phenols are orientated *para* to a chlorine atom. *Para* hydroxylation is not possible in *p*-dichlorobenzene and in this case the monophenol formed has the only orientation possible. With *o*- and *m*-dichlorobenzene small amounts of a second monophenol are produced, and in both cases the hydroxyl group is orientated *meta* to a chlorine atom, although in *o*-dichlorobenzene it is also *ortho* to chlorine. The two monophenols of *o*-dichlorobenzene may be produced by different reactions since the 3:4-dichlorophenol reaches its peak excretion before the 2:3-phenol (Azouz *et al.* 1955). This could be explained by postulating the formation of two dihydrodiols formed at different rates and possibly decomposing at different rates; the first, 1:2-dihydro-4:5-dichlorobenzene-1:2-diol (I), would dehydrate to the 3:4-phenol only, whereas the second, 1:2-dihydro-3:4-dichlorobenzene-1:2-diol (II), could yield both the 2:3- and 3:4-phenols possibly in equal amounts, thus:



Dehydrogenation of I and II respectively could also yield the 4:5- and 3:4-dichlorocatechols found in *o*-dichlorobenzene urine.

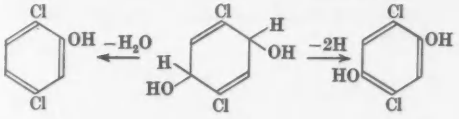
The occurrence of 2:4- and 3:5-dichlorophenols as metabolites of *m*-dichlorobenzene could be explained by a similar mechanism, the intermediate diol in this case being 1:2-dihydro-3:5-dichlorobenzene-1:2-diol (III). This diol could be expected to yield mainly 2:4-dichlorophenol in which the hydroxyl group is *ortho* or *para* to the chlorine, and a smaller amount of the 3:5- isomer, which is in fact what is found.



Dehydrogenation of III would yield the catechol of *m*-dichlorobenzene urine, namely, 3:5-dichlorocatechol. The orientations of the phenols produced *in vivo* from *o*- and *m*-dichlorobenzene could thus be satisfactorily explained by postulating the formation of 1:2-dihydro-1:2-diols, although direct hydroxylation reactions may also be involved. We

looked for dihydrodiols but failed to isolate any. However, it may be recalled that a dihydrodiol of monochlorobenzene has been isolated from urine (Smith, Spencer & Williams, 1950).

In the case of *p*-dichlorobenzene the formation of a 1:4-dihydro-1:4-diol (IV) would explain the metabolites better than a 1:2-dihydro-1:2-diol, since a quinol is formed rather than a catechol, thus:



(IV)

Direct hydroxylation, however, could explain these reactions of the *para* compound equally well. It is to be noted that quinols were not detected as metabolites of *o*- and *m*-dichlorobenzenes.

It is to be expected that the output of total phenols would be equal to the sum of the glucuronide and ethereal sulphates. But an examination of Table 4 shows that the total phenols fall short of the total *O*-conjugates (*ortho*: *O*-conjugates, 69%, phenols, 43%; *meta*: *O*-conjugates, 43%, phenols, 28%; *para*: *O*-conjugates, 63%, phenols, *ca.* 41%). We believe these differences are due in part to loss of phenols during acid hydrolysis possibly due to their interaction with a variety of compounds which occur in urine, and also to the errors in the methods of estimating glucuronic acid and ethereal sulphates which are liable to an error of ±10%.

Orientation of the mercapturic acids

When monochlorobenzene is fed to animals the mercapturic acid excreted contains an *N*-acetylcysteine group which is *para* to the chlorine atom. This is also true for fluoro-, bromo- and iodobenzene. An examination of the mercapturic acid fraction of chlorobenzene urine from rabbits failed to reveal any of the *ortho* or *meta* isomer. The small mercapturic acid fraction of *o*-dichlorobenzene contains only one such acid and this has been proved to be 3:4-dichlorophenylmercapturic acid by conversion into 3:4:3′:4′-tetrachlorodiphenyldisulphide, which was isolated and identified. (The mercapturic acid is probably of the L- series, since all naturally formed mercapturic acids belong to this configuration.)

Similarly, it has been proved that *m*-dichlorobenzene yields only 2:4-dichlorophenylmercapturic acid, and this in small amounts. Since *p*-dichlorobenzene does not yield a mercapturic acid, it appears that, in the mono- and di-chlorobenzene series, acetylcysteine conjugation only occurs in a free position which is *para* to a chlorine atom. Furthermore, it appears that the extent of this conjugation diminishes as the number of chlorine atoms substituted into the benzene ring increases (at least up to three) (see also Table 4), since other work in this laboratory has shown that the three isomeric trichlorobenzenes do not result in the excretion of appreciable amounts of mercapturic acid, although free *para* positions are available in all three (Jondorf, Parke & Williams, unpublished results). Mercapturic acid formation in polychlorinated benzenes however is known to occur, since Bray, Hybs, James & Thorpe (1953) found 2:3:5:6-tetrachloronitrobenzene to yield 2:3:5:6-tetrachlorophenylmercapturic acid in large amounts in rabbits, the nitro group being eliminated. Other work in this laboratory has shown that 1:2:4:5-tetrachlorobenzene does not form a mercapturic acid in rabbits in amounts greater than 0·8% of the dose, which is the limit of our method. This fact makes the mechanism of mercapturic acid formation from 2:3:5:6-tetrachloronitrobenzene highly interesting and suggests substitution of the nitro group by acetylcysteine (cf. Bray *et al.* 1953).

SUMMARY

1. *m*-Dichlorobenzene is oxidized in rabbits mainly to 2:4-dichlorophenol which is excreted conjugated with glucuronic and sulphuric acid. Small amounts of conjugated 3:5-dichlorophenol and 3:5-dichlorocatechol are also excreted.

2. *m*-Dichlorobenzene is excreted to a minor extent as 2:4-dichlorophenylmercapturic acid.

3. The excretion of these metabolites is slow and is virtually complete in about 5 days after dosing.

4. The mercapturic acid formed from monochlorobenzene in rabbits has been carefully examined and it has been shown to contain only the *para* isomer; the *o*- and *m*-chlorophenylmercapturic acids are not formed.

5. These results have been compared with those from chlorobenzene and *o*- and *p*-dichlorobenzene and discussed.

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Studies in Detoxication

64. THE SYNTHESIS OF THE ISOMERIC DICHLOROPHENYLMERCAPTURIC ACIDS

By D. V. PARKE
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

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Recent work in this laboratory on the metabolism of the *o*-, *m*- and *p*-dichlorobenzenes in rabbits (Azouz, Parke & Williams, 1953, 1955; Parke & Williams, 1955) has shown that mercapturic acids are minor metabolites (*ca.* 5% of the dose) of the *ortho* and *meta* isomers. In order to identify these acids, the synthesis of the six isomeric L-dichlorophenylmercapturic acids became necessary. The present paper describes the synthesis and properties of these compounds and the corresponding dichlorophenyl-L-cysteines. At the same time, the mercapturic acid fraction obtained after feeding chlorobenzene to rabbits was examined chromatographically to ascertain whether the *o*- and *m*-chlorophenylmercapturic acids were present in addition to the principal metabolite, *p*-chlorophenylmercapturic acid (Spencer & Williams, 1950*a*, *b*). The *o*- and *m*-chlorophenylmercapturic acids and the corresponding chlorophenyl-L-cysteines were therefore synthesized. Since the completion of this work, a paper by West & Mathura (1954) on the synthesis of these latter compounds has appeared.

EXPERIMENTAL

Melting points are corrected.

Materials. L-Cystine was purified by dissolving 10 g. in 200 ml. N-H_2SO_4, filtering, and reprecipitating with a saturated solution of ammonium acetate. After 1 hr. at 0°, the cystine was filtered off, washed with water, and dried in air. 2-Chloroaniline (b.p. 209°) and 3-chloroaniline (b.p. 230°) were purchased and purified by distillation. 2:3-Dichloroaniline (m.p. 24°), 2:4-dichloroaniline (m.p. 64°), 2:5-dichloroaniline (m.p. 52°), and 3:4-dichloroaniline (m.p. 72°) were purchased and were purified by distillation in steam. 2:6-Dichloroaniline (m.p. 39°) was prepared from 3:5-dichlorosulphanilamide as described by Seikel (1944). 3:5-Dichloroaniline (m.p. 50°) was prepared from 3:5-dichloronitrobenzene (Roberts & Turner, 1927) according to Dyson, George & Hunter (1926), and was purified by steam distillation.

Preparation of dichlorophenylmercapturic acids. The isomeric *S*-dichlorophenyl-L-cysteines were synthesized from L-cystine (5 g.) and the corresponding dichloroanilines (7·5 g.) according to Parke & Williams (1951). Improved yields were obtained if the solution of cysteine cuprous mercaptide was filtered hot, to remove traces of copper oxides, before cooling to 0° and coupling with the diazotized dichloroanilines. The *S*-dichlorophenyl-L-cysteines (yield, 0·5–1·2 g.) were isolated by repeated extraction with 50% aqueous ethanol, in which cystine is sparingly soluble. These compounds were acetylated in pyridine with acetic anhydride to the corresponding mercapturic acids (Zbarsky & Young, 1943), which were then recrystallized from water or aqueous ethanol. A more efficient procedure for the isolation of pure dichlorophenyl-L-cysteines in quantity was to acetylate the crude dichlorophenylcysteine–cystine mixture to give the L-dichlorophenylmercapturic acid (yield, 1·5–4·5 g.) which was isolated, purified and subsequently hydrolysed with acid to the corresponding dichlorophenyl-L-cysteine.

Acid hydrolysis of dichlorophenylmercapturic acids. The pure L-dichlorophenylmercapturic acids (1 g.) were hydrolysed to the corresponding *S*-dichlorophenyl-L-cysteines by heating under reflux with 50 ml. 2N-H_2SO_4 for 1–2 hr. 2:5-Dichlorophenylmercapturic acid was more resistant to acid hydrolysis than the other isomers, and 50 ml. of 5N-H_2SO_4 was used in this case. The hydrolysed solutions were neutralized with 2N ammonia, and then adjusted to pH 5 with 2N acetic acid. The precipitated dichlorophenylcysteines were filtered off and recrystallized from aqueous ethanol. In no instance was there any evidence of racemization of the dichlorophenyl-L-cysteines, since the products obtained by acid hydrolysis of the mercapturic acids were

Table 1. *Properties of the* L-*chlorophenyl- and* L-*dichlorophenyl-mercapturic acids*

Isomer	Yield from 5 g. cystine (g.)	M.p. (°)	Solubility in water (%)	$[\alpha]_D^{22}$* in ethanol (c, 1·0) (°)	pK	Found (%) C	H	N	Cl	SH	Equiv.
Chlorophenylmercapturic acids											
ortho (rhombs)†	2·5	151	0·10	− 14	3·4	48·3	4·2	5·2	13·4	11·9	265
meta (rhombs)†	4·0	165	0·14 } at 10°	− 19	3·5	48·2	4·8	4·8	13·2	12·5	271
para	—	153‡	0·06	− 20‡	3·8§	—	—	—	—	—	—
			($C_{11}H_{12}O_3NClS$ requires			48·3	4·4	5·1	13·0	12·1	274)
Dichlorophenylmercapturic acids											
2:3- (needles)	1·8	146		0	3·7	43·0	3·8	4·5	23·4	10·1	314
2:4- (needles)	3·2	160	— —	− 9	3·5	42·9	3·7	4·5	23·1	10·0	317
2:5- (rosettes of needles)	3·8	156		− 9	3·8	43·4	3·4	4·3	22·6	10·5	311
2:6- (rhombs)	4·5	191	0·15 } at 20°	+ 10‖	4·0	43·0	3·5	4·6	23·2	10·7	296
3:4- (needles)	1·5	149	0·04 }	− 8	3·4	42·8	3·6	4·3	22·6	10·0	320
3:5- (needles)	2·0	150	—	− 11	3·5	42·7	3·4	4·4	22·4	10·3	317
			($C_{11}H_{11}O_3NCl_2S$ requires			42·9	3·6	4·5	23·0	10·4	308)

* Error ± 2°.
† West & Mathura (1954) give for *ortho* compound, m.p. 146·5–147·5° and $[\alpha]_D^{28}$ − 6·1° and for *meta* compound m.p. 160·5–161° and $[\alpha]_D^{28}$ − 11°.
‡ Figures quoted from Parke & Williams (1951).
§ Figure quoted from Robinson *et al.* (1953).
‖ $[\alpha]_D^{20}$ + 13°.

identical with those obtained directly from L-cystine and the diazotized dichloroanilines. The properties of the *dichlorophenylmercapturic acids* and *dichlorophenylcysteines*, and the *o*- and *m*-chlorophenylmercapturic acids and chlorophenylcysteines are given in Tables 1 and 2.

Alkaline hydrolysis of the mercapturic acids. The SH content of the mercapturic acids after alkaline hydrolysis was determined by titration with 0·01 N-I_2 after heating at 100° for 40 min. in N-NaOH (Parke & Williams, 1951). All compounds were quantitatively decomposed into the corresponding thiophenols by this treatment, and may therefore be estimated by the iodometric procedure (cf. Stekol, 1936).

Determination of pK_a. The apparent dissociation constants of the mercapturic acids in aqueous solution were determined in the usual manner from the potentiometric titration curves (cf. Robinson, Smith & Williams, 1953) (see Table 1).

Fig. 1. The ultraviolet absorption spectra of the L-chlorophenylmercapturic acids in ethanol: *A*, *ortho* ($\lambda_{max.}$ 255 mμ., ε 8600); *B*, *meta* ($\lambda_{max.}$ 256 mμ., ε 8000); *C*, *para* ($\lambda_{max.}$ 260–261 mμ., ε 11 200) (Parke & Williams, 1951).

Fig. 2. The ultraviolet absorption spectra of the chlorophenyl-L-cysteines in 0·1 N-NaOH (——): *A*, *ortho* ($\lambda_{max.}$ 252–253 mμ., ε 8400); *B*, *meta* ($\lambda_{max.}$ 257 mμ., ε 9500); *C*, *para* ($\lambda_{max.}$ 260 mμ., ε 12 600); and in 0·1 N-HCl (– – –); *A*, *ortho* ($\lambda_{max.}$ 250 mμ., ε 5900); *B*, *meta* ($\lambda_{max.}$ 256 mμ., ε 7000); *C*, *para* ($\lambda_{max.}$ 257 mμ., ε 10 600).

Table 2. *Properties of the* S*-chlorophenyl- and* S*-dichlorophenyl-*L*-cysteines*

Isomer	Yield* (g.)	M.p. (decomp.) (°)	$[\alpha]_D^{20}$† in 0·1N-NaOH (*c*, 1·0) (°)	Found (%) C	H	N	Cl
*Chlorophenyl-*L*-cysteines*							
ortho (plates)	1·1	184	+4	—	—	6·2	14·9
meta (needles)	1·5	183	+14	—	—	6·2	14·7
para	—	190‡	+18‡	—	—	—	—
		($C_9H_{10}O_2NClS$ requires				6·1	15·3)
*Dichlorophenyl-*L*-cysteines*							
2:3- (plates)	0·8	184	+12	40·3	3·3	5·2	26·2
2:4- (plates)	1·0	180	+15	—	—	4·9	26·0
2:5- (needles)	1·2	178	+10	—	—	5·2	26·0
2:6- (needles)	1·5	175	−15	—	—	5·4	26·6
3:4- (plates)	0·5	177	+17	40·7	3·2	5·3	26·3
3:5- (plates)	0·8	181	+14	—	—	5·2	27·1
		($C_9H_9O_2NCl_2S$ requires		40·6	3·4	5·3	26·6)

* Obtained directly from L-cystine (5 g.). † Error ±2°.
‡ Quoted from Parke & Williams (1951).

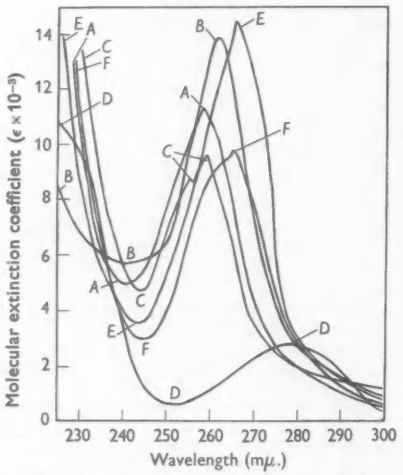

Fig. 3. The ultraviolet absorption spectra of the L-dichlorophenylmercapturic acids in ethanol: *A*, 2:3- ($\lambda_{max.}$ 258 mμ., ε 11 200); *B*, 2:4- ($\lambda_{max.}$ 261–262 mμ., ε 13 800); *C*, 2:5- ($\lambda_{max.}$ 256 and 259 mμ., ε 8600 and 9600); *D*, 2:6- ($\lambda_{max.}$ 278 mμ., ε 2800); *E*, 3:4- ($\lambda_{max.}$ 265 mμ., ε 14 500); *F*, 3:5- ($\lambda_{max.}$ 265 mμ., ε 9800).

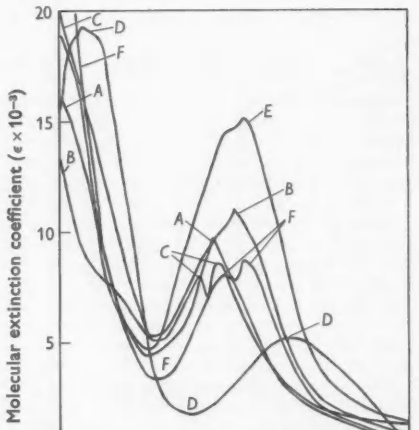

Fig. 4. The ultraviolet absorption spectra of the dichlorophenyl-L-cysteines in 0·1N-NaOH: *A*, 2:3- ($\lambda_{max.}$ 255 mμ., ε 9700); *B*, 2:4- ($\lambda_{max.}$ 260 mμ., ε 10 800); *C*, 2:5- ($\lambda_{max.}$ 252 and 256–257 mμ., ε 8000 and 8400); *D*, 2:6- ($\lambda_{max.}$ 225 and 272–273 mμ., ε 19 300 and 5200); *E*, 3:4- ($\lambda_{max.}$ 262 mμ., ε 15 000); *F*, 3:5- ($\lambda_{max.}$ 258 and 262 mμ., ε 8000 and 8700).

Light absorption. The ultraviolet absorption spectra of the mercapturic acids in ethanol, and of the phenylcysteines in 0·1N-NaOH and in 0·1N-HCl, were determined with a Unicam spectrophotometer, Model SP. 500 (see Figs. 1–5).

Solubility. The chlorophenylmercapturic acids and the dichlorophenylmercapturic acids are colourless compounds, soluble in ethanol, less soluble in ether and $CHCl_3$, and only sparingly soluble in cold water. The solubilities of the dichlorophenylmercapturic acids in ether are equal to or greater than those in $CHCl_3$, so that ether could be used for the extraction of these compounds from urine. Chloroform is usually the solvent used for extracting mercapturic acids from urine.

DISCUSSION

The six isomers of *S*-dichlorophenyl-L-cysteine have been prepared from cystine in yields of about 10%, and have been acetylated to the corresponding L-dichlorophenylmercapturic acids in almost theoretical yields. The pK_a values of these mercapturic acids all lie between 3·4 and 4·0, which is in agreement with the work of Robinson *et al.* (1953). The

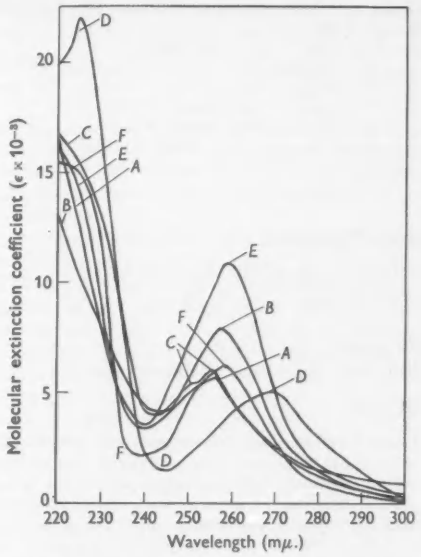

Fig. 5. The ultraviolet absorption spectra of the dichlorophenyl-L-cysteines in 0·1 N-HCl: *A*, 2:3- ($\lambda_{max.}$ 256 mμ., ϵ 5700); *B*, 2:4- ($\lambda_{max.}$ 257 mμ., ϵ 7900); *C*, 2:5- ($\lambda_{max.}$ 250 and 255–256 mμ., ϵ 5400 and 5700); *D*, 2:6- ($\lambda_{max.}$ 225 and 268–270 mμ., ϵ 22000 and 5000); *E*, 3:4- ($\lambda_{max.}$ 258–260 mμ., ϵ 10750); *F*, 3:5- ($\lambda_{max.}$ 258 mμ., ϵ 6250).

effect of the chlorine atoms in the aromatic ring on the pK_a value is small, as might be expected from a comparison with the β-(chlorophenyl)-propionic acids (Dippy, 1939), where the introduction of chlorine increases the dissociation of β-phenylpropionic acid (pK_a, 4·66) by only a slight degree (*ortho*-chlorophenylpropionic acid, pK_a 4·58; *meta*-, pK_a 4·59; *para*-, pK_a 4·61). The pK_a values of the dichlorophenylmercapturic acids are, moreover, affected by the relative positions of the two chlorine atoms to the acetylcysteine moiety, the pK_a increasing as the direction of the resultant dipole of the chlorine atoms changes from the *para* to the *ortho* position.

The specific optical rotations of the dichlorophenyl-L-cysteines also decrease in this same order. With the mercapturic acids, however, the specific rotation is made more positive by the presence of *ortho*-substituted chlorine, viz., L-phenylmercapturic acid, $[\alpha]_D - 21°$; 2-chlorophenylmercapturic acid, $[\alpha]_D - 14°$; 2:3-dichlorophenylmercapturic acid, $[\alpha]_D$ 0°; 2:6-dichlorophenylmercapturic acid, $[\alpha]_D + 10°$; and 2:3:5:6-tetrachlorophenylmercapturic acid, $[\alpha]_D + 33°$ (Bray, Hybs, James & Thorpe, 1953). The ultraviolet absorption maxima of the dichlorophenylcysteines and the dichlorophenylmercapturic acids show a slight decrease in wavelength as the resultant chlorine dipoles change from the *para* to the *ortho* position, but an anomaly is provided by the 2:6- isomers, which have an absorption spectrum similar to that of non-ionized 2:6-dichlorophenol (absorption maximum in 0·1 N-HCl, 2760 Å; ϵ, 2250; cf. Parke & Williams, 1955). This suggests that the two chlorine atoms vicinal to the cysteine or acetylcysteine moiety cause steric inhibition of uniplanarity of the molecule, which results in a change both in wavelength and intensity of the absorption band (cf. Braude, Sondheimer & Forbes, 1954). The solubilities of the dichlorophenylmercapturic acids in water also increase steadily from the 3:4- isomer to the 2:6- isomer and the 2:5- and 2:6-dichlorophenylcysteines are noticeably more soluble than the other isomers in 50% aqueous ethanol. A similar relationship between optical activity, dissociation constants, and solubilities has been observed by Pauly (1921) in a series of (−)-menthol esters.

SUMMARY

1. The six isomeric *S*-dichlorophenyl-L-cysteines and the corresponding L-dichlorophenylmercapturic acids have been prepared in about 10% yield from L-cystine.

2. The dichlorophenylmercapturic acids are completely hydrolysed by heating in N-NaOH at 100° for 40 min., and may be estimated iodometrically.

3. The apparent pK_a values of *o*- and *m*-chlorophenylmercapturic acids and the dichlorophenylmercapturic acids have been measured, and range from 3·4 to 4·0.

4. The ultraviolet absorption spectra of the *o*- and *m*-chlorophenylmercapturic acids, the isomeric dichlorophenylmercapturic acids and the corresponding *S*-phenylcysteines have been determined. The absorption maxima are related to the relative positions of the aromatic halogen atoms. The wavelength and intensity of the absorption maxima of L-2:6-dichlorophenylmercapturic acid and the corresponding *S*-phenylcysteine are anomalous, and indicate steric inhibition of molecular uniplanarity.

I am grateful to Professor R. T. Williams for his interest in the work. The expenses of this work were in part defrayed by a grant to Professor Williams from the Medical Research Council.

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Tissue Fractionation Studies

3. FURTHER OBSERVATIONS ON THE BINDING OF ACID PHOSPHATASE* BY RAT-LIVER PARTICLES

BY FRANÇOISE APPELMANS AND C. DE DUVE
Department of Physiological Chemistry, University of Louvain, Belgium

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A considerable part of the unspecific acid phosphatase of rat liver is bound to cytoplasmic granules which are recovered mainly with the mitochondria, but also to some extent with the microsomes (Berthet & Duve, 1951). Recent experiments have shown that these granules form a separate group, entirely distinct from the cytochrome oxidase-containing mitochondria and from the glucose 6-phosphatase-bearing microsomes (Duve, Gianetto, Appelmans & Wattiaux, 1953; Appelmans, Wattiaux & Duve, 1955).

Fresh preparations show very little enzymic activity when assayed under conditions which preserve their morphological integrity; they are easily activated by a number of treatments, which all cause the simultaneous release of the enzyme in soluble form. Investigations bearing on this phenomenon led to the conclusion that acid phosphatase is retained within the granules by a membrane-like barrier, impermeable to sucrose and glycerophosphate, but not to a number of other substances, such as glycerol, glucose and various inorganic salts (Berthet, Berthet, Appelmans & Duve, 1951). The granules appeared to behave as typical osmotic systems.

The present paper deals with a more detailed study of the facts observed previously. In general, the new results afford additional support to the earlier conclusions. However, some of the concepts on which these were based have had to be re-examined.

METHODS

Washed mitochondria were isolated from 0·25M sucrose homogenates of rat liver by a slight modification of the method described previously (Berthet & Duve, 1951). The pressing of the liver through a wire mesh was omitted and higher field-time values were used to ensure a better yield of phosphatase-containing granules. Using a Spinco Model L preparative ultracentrifuge, with rotor no. 40 ($r_{max.}$ = 8·1 cm., $r_{min.}$ = 4·8 cm.), the 1:10 cytoplasmic extract, free of nuclei, was usually centrifuged at 160000 *g*-min.; ($S_{min.}$ = 3500 *S*); the resuspended particles were washed twice at 130000 *g*-min. ($S_{min.}$ = 4400 *S*). (The use of the composite unit *g*-min. has been proposed by Duve & Berthet (1953). It is necessitated in the present case by the fact that the centrifugings are performed essentially by means of up-and-down runs, full advantage being taken of the force developed during acceleration and deceleration. The term $S_{min.}$ refers to the sedimentation constant, expressed in Svedberg units (10^{-13} sec.), of the lightest spherical particles which are completely sedimented under the conditions used.)

Acid phosphatase was measured as before, with β-glycerophosphate as substrate. Various commercial preparations were used, after it had been ascertained by means of periodate titration that they contained no more than 5% of the α isomer (which, unlike the β isomer, is significantly hydrolysed by the microsomal glucose 6-phosphatase).

Most of the assays were carried out in 10 min. at pH 5 and 37°, or for longer periods at pH 6·1 and 0°. As already shown (Berthet & Duve, 1951), little thermal activation occurs under these conditions, allowing for a selective estimation of the amount of free enzyme present in the incubation mixture. The total enzyme content of the preparations was measured in a similar manner on suspensions previously treated for 3 min. in a Waring Blendor (usually after tenfold dilution with distilled water), care being taken to prevent excessive heating of the preparation during this treatment. The reaction was stopped by the addition of 8% (w/v) trichloroacetic acid and inorganic P estimated on the filtrates by the method of Fiske & Subbarow (1925).

RESULTS

Osmotic activation

As reported previously (Berthet *et al.* 1951), treatment of granules with distilled water causes a rapid release of their acid phosphatase activity. The same

* Part 2 of this series: Berthet, Berthet, Appelmans & Duve (1951).

phenomenon occurs, but in a more progressive manner, when the granules are kept at 0° in 0·25 M solutions of various ionized or non-ionized substances. The rate of release at 0° is negligible in 0·25 M sucrose or β-glycerophosphate, or in media containing 0·25 M sucrose in addition to a substance such as NaCl or KCl, which alone is unable to prevent the activation. The osmotic properties of the granules were deduced from the results of these experiments.

In most of the investigations mentioned above, the release of the phosphatase was followed by determining the amount of unsedimentable enzyme present after centrifuging off the particulate material at high speed. Owing to adsorption phenomena, this method does not measure the total activity set free. This drawback has been overcome in the present studies by assaying the free activity of the preparations in the presence of the particles. In addition, advantage has been taken of a technique involving the graded osmotic lysis of the granules, to investigate some of the finer aspects of this phenomenon.

Graded osmotic lysis. Fig. 1 shows the influence of sucrose concentration on the rate of liberation of inorganic P by granules incubated at pH 6·1 and 0° in the presence of 0·0125 M sodium β-glycerophosphate. The reaction proceeds linearly with time in all cases, but at a rate which increases with decreasing sucrose concentration. Thus each decrease in sucrose concentration causes the rapid release of a finite fraction of bound enzyme, and no additional activation occurs during the subsequent incubation.

The general shape of the activation curve is shown in Fig. 2, in which the rates, expressed in percentage of the maximal rate achieved by the blender-treated preparation, are plotted against sucrose concentration. This graph includes the results of a parallel experiment made in the presence of 10^{-4} M 2:4-dinitrophenol. It is seen that this compound, which has been found to activate the latent ATPase of mitochondria (Hunter, 1951; Potter & Recknagel, 1951; Lardy & Wellman, 1953), is without effect on the osmotic activation of acid phosphatase.

Osmotic protection by glycerophosphate. In a similar experiment, run at two different concentrations of glycerophosphate, the curve obtained with the higher substrate concentration was parallel to the other, but shifted to the left by an abscissa value corresponding to a difference in sucrose concentration of approximately 0·03 M (Fig. 3). Since this shift is due to the presence of 0·0125 M glycerophosphate in excess, it follows that 12·5 μmoles of glycerophosphate show the same protective effect on the granules as 30 μmoles of sucrose. At pH 6·1, 12·5 μmoles of glycerophosphate ($pK_2 = 6·37$, according to Kiessling, 1934) are equilibrated by 17·1 μequiv. of sodium, and will create an osmotic pressure equal to that of 29·6 μmoles of sucrose dissolved in the same volume. The data of Fig. 3, therefore, demonstrate in a quantitative manner that the protection afforded by glycerophosphate is of osmotic nature, a conclusion which had already

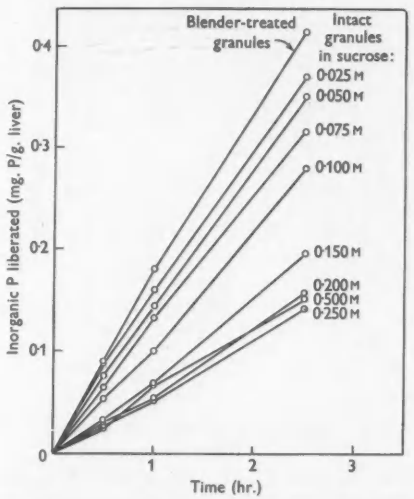

Fig. 1. Influence of sucrose concentration on the acid phosphatase activity of a mitochondrial preparation. Incubation at 0° in 0·0125 M sodium β-glycerophosphate, pH 6·1, and sucrose at concentration indicated.

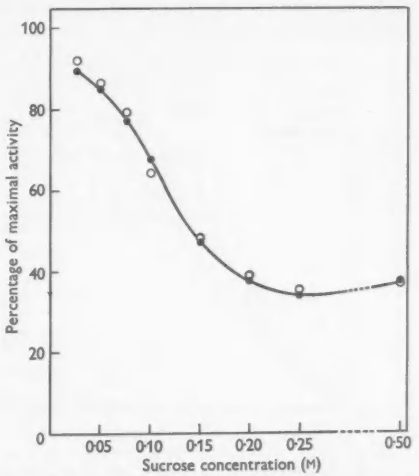

Fig. 2. Osmotic activation curve. ●, data of Fig. 1; ○, duplicate experiment, with 10^{-4} M 2:4-dinitrophenol.

been drawn from earlier experiments in which large amounts of glycerophosphate were used.

Transient protection by monovalent salts. The effect of inorganic salts has also been studied by the new technique. Fig. 4, which should be compared with Fig. 1, shows the rate of liberation of inorganic P when 0·15 M-NaCl is present in addition to the other components. The rates are initially equal, but increase progressively in the samples which are hypotonic with respect to sucrose, to reach finally the value which would have obtained immediately if no NaCl had been present. Obviously, the salt affords only a transient osmotic protection, which

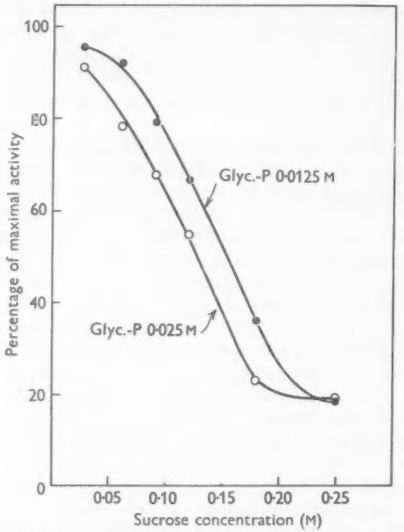

Fig. 3. Influence of glycerophosphate concentration on the osmotic release of acid phosphatase. Experimental conditions as in Figs. 1 and 2, with substrate concentrations indicated.

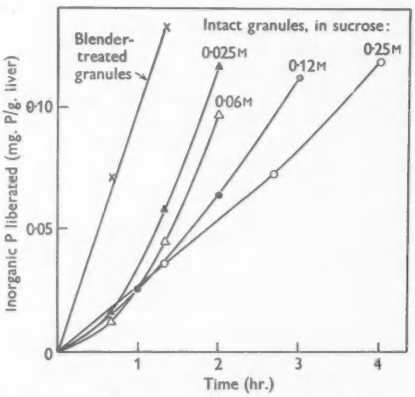

Fig. 4. Transient osmotic protection by NaCl. Experimental conditions as in Fig. 1, but with 0·15 M-NaCl in all samples.

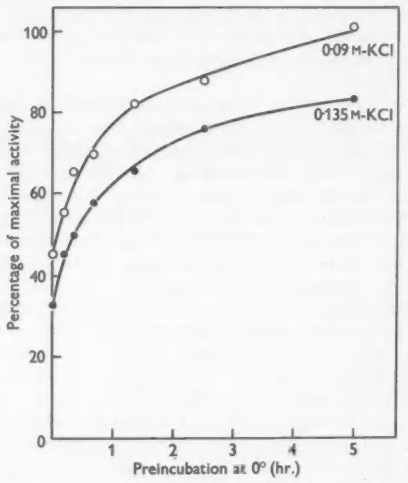

Fig. 5. Osmotic activation in solutions of KCl. Preincubation at 0° and pH 6·1 (0·002 M cacodylate buffer) in KCl at concentration indicated on graph. Free acid phosphatase measured in 10 min. at pH 5 and 37° in 0·05 M glycerophosphate, 0·05 M acetate buffer, 0·25 M sucrose and KCl at the concentration used for the preincubation.

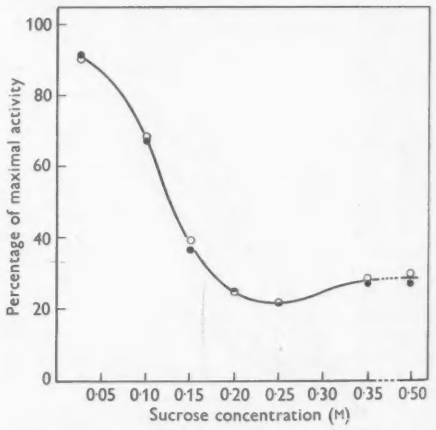

Fig. 6. Irreversibility of osmotic activation. Preincubation 30 min. at 0° in sucrose concentration indicated on the abscissa. Free acid phosphatase measured in 10 min. at 37° in 0·006 M glycerophosphate adjusted to pH 5 and sucrose at same concentration as during preincubation (●) or 0·25 M (○).

disappears as the concentration within the granules becomes equilibrated by diffusion with that in the medium.

In the experiment of Fig. 5, the granules were kept at 0° and pH 6·1 in two different concentrations of KCl, sucrose being 0·025 M in the mixture. Free acid phosphatase was measured at regular intervals at pH 5 and 37°. The substrate added contained KCl at the same concentration to prevent the bursting of salt-enriched granules (Berthet *et al.* 1951). The results are consistent with the hypothesis that diffusion is the factor limiting the rate of activation when the granules are suspended in salt solutions. However, attempts to measure quantitatively the permeability constant of the granules to various salts in experiments of this type failed, owing to the difficulty of establishing the exact relationship between the penetration of salt and the release of enzyme.

Irreversibility of osmotic activation. In order to study the possible degree of reversibility of the activation which occurs in hypotonic media, duplicate samples of granules were preincubated for 30 min. at 0° in the presence of different sucrose concentrations, following which the free enzyme was estimated at pH 5 and 37°. Dilute glycerophosphate was used, in order to minimize the contribution of the substrate to the osmotic pressure. In one series, the sucrose concentration was kept constant during the preincubation period and the actual assay. In the other, sufficient sucrose was added with the substrate to make its concentration 0·25 M in all cases during the enzyme test. As is shown in Fig. 6, this procedure did not decrease the amount of enzyme made available by hypotonic conditions and it may be concluded that osmotic activation is entirely irreversible.

Reproducibility of osmotic activation. In Fig. 7, the results of six different experiments have been plotted against the minimum effective osmotic pressure of the medium to which the granules had been exposed. Some differences appear to exist between individual preparations with respect to their resistance to osmotic disruption. However,

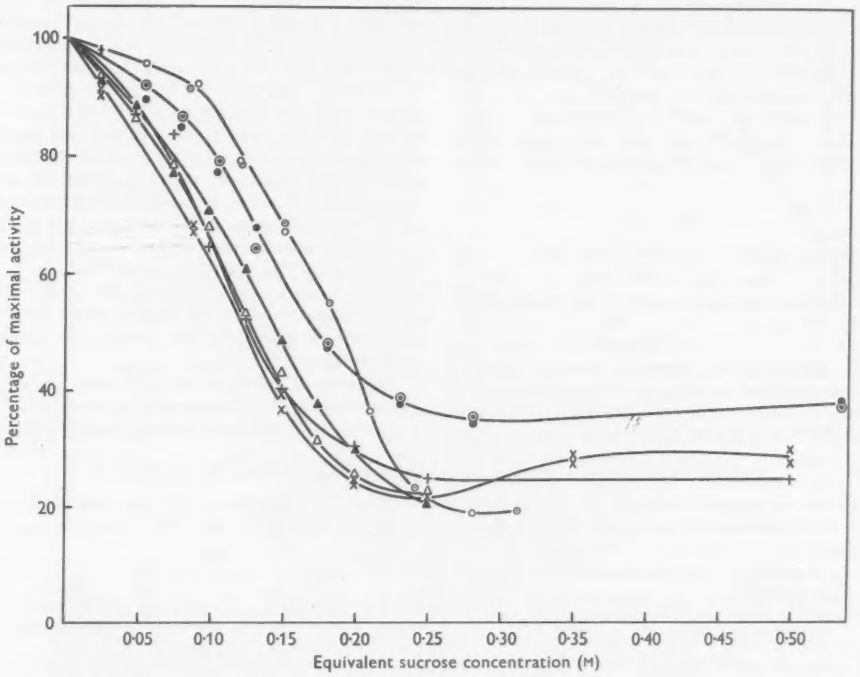

Fig. 7. Reproducibility of osmotic activation. ●, experiment of Fig. 2; ⊙, same with 10^{-4} M dinitrophenol; ○, experiment of Fig. 3, 0·0125 M glycerophosphate; ⊙, experiment of Fig. 3, 0·025 M glycerophosphate; ×, experiment of Fig. 6; △▲+, data of three experiments, in which the free activity was measured in 10 min. at 37° and pH 5 in 0·25 M sucrose, after 15 min. exposure at 0° to varying osmotic pressures. For the experiments of Figs. 2 and 3, the glycerophosphate present has been considered as iso-osmolar sucrose in the abscissa.

differences in technique may have to be considered as well, for the two upper curves in Fig. 7 were obtained at 0° and pH 6·1, whereas the four lower ones were established at 37° and pH 5. As will be shown below, there is some evidence of a greater contribution of the internal enzyme to the free activity at pH 6·1.

Thermal activation

Berthet *et al.* (1951) have shown that granules incubated at 38° in isotonic sucrose progressively release acid phosphatase in unsedimentable form. However, the true amount of enzyme set free was not measured and no attention was paid to the pH of the incubation mixture. It was therefore decided to repeat these experiments under more controlled conditions.

Effect of pH. Equal quantities of a mitochondrial suspension were incubated at 37° in individual test tubes, containing 0·25 M sucrose and either 0·1 M cacodylate buffer, pH 6·1, or 0·1 M acetate buffer, pH 5. The free activity was measured at regular intervals by adding equal volumes of a mixture containing 0·1 M glycerophosphate adjusted to the same pH and 0·25 M sucrose, and incubating for 10 min. Similar tests were made on blender-treated preparations. Fig. 8 shows the results of a representative experiment of this type.

It is seen that the activation proceeds more rapidly at pH 5 than at pH 6·1. This difference is not, however, apparent during the early phase of the process, and the initial free activity of the preparation is actually higher at pH 6·1 than at pH 5. As will be shown later (Fig. 9) this discrepancy is explained by the difference in the pH/activity curves of the free and total activities and it may be concluded that thermal activation is at all times more rapid at pH 5 than at pH 6·1.

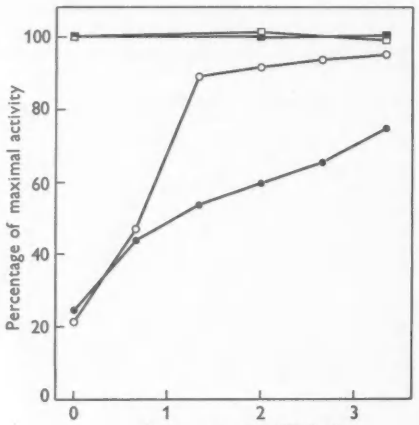

Fig. 8. Influence of pH on thermal activation. For experimental conditions, see text. ○, intact granules, pH 5; ●, intact granules, pH 6·1; □, blender-treated granules, pH 5; ■, blender-treated granules, pH 6·1.

Kinetic properties of the free enzyme

As shown in this and previous papers, homogenates or mitochondrial preparations always contain a certain amount of free phosphatase, representing usually 10–20 % of the total activity or sometimes more if the granules have been damaged in the course of isolation. Only part of this activity is recovered in soluble form when the granules are centrifuged off, but strong evidence was obtained that the part which remains attached to the sediment is fundamentally different from the native bound form and must be considered as free enzyme secondarily adsorbed to the surface of the granules, or perhaps also partly trapped within 'ghosts' of injured granules (Berthet *et al.* 1951). In the present study an attempt has been made to compare the kinetics of the free activity of mitochondrial suspensions with those of completely disrupted preparations.

Influence of substrate concentration. Raising the concentration of glycerophosphate, even within the range where the enzyme is almost saturated, hardly

Table 1. *Influence of substrate concentration on free and total activity at* pH 5

Free activities measured on intact granules, incubated 10 min. at 37° in 0·25 M sucrose and 0·05 M acetate buffer, pH 5; total activities measured similarly on blender-treated granules. Results expressed as μg. P/10 min./g. liver.

Glycerophosphate concn. (m-moles/l.)	Expt. 1			Expt. 2		
	Total activity	Free activity	Free activity (% of total)	Total activity	Free activity	Free activity (% of total)
3	300	44·6	14·9	297	40·3	13·6
6	460	54·5	11·8	435	51·7	11·9
12	600	62	10·3	623	67·5	10·8
24	745	71·3	9·6	802	87·8	10·9
36	855	82·1	9·6	920	102	11·1
60	935	89	9·5	1028	120	11·7
90	955	97·5	10·2	1070	131	12·2
120	958	99	10·3	1082	133	12·3

causes a greater proportion of the total activity to appear as 'free' enzyme. This fact, which is shown clearly in Table 1, furnishes strong corroborative evidence of the essential impermeability of the granules to glycerophosphate. A slight relative increase of free activity is noted at very low substrate concentrations, suggesting that glycerophosphate may, by itself, exert some kind of action on the granules. This action could possibly affect their permeability, but a more likely explanation is that it consists simply in a protective effect against thermal activation during the assay.

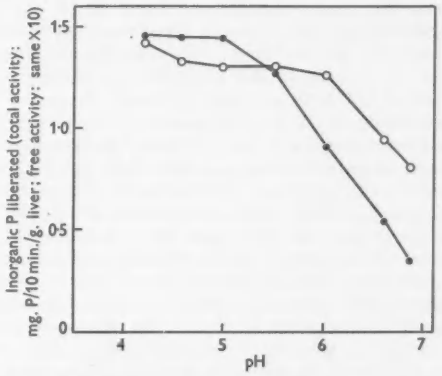

Fig. 9. Influence of pH on free and total activity. Assays performed in 10 min. at 37°, in 0·05 M glycerophosphate, 0·05 M acetate and 0·05 M cacodylate, adjusted to desired pH. ○, intact granules, assayed in 0·25 M sucrose; ●, blender-treated granules.

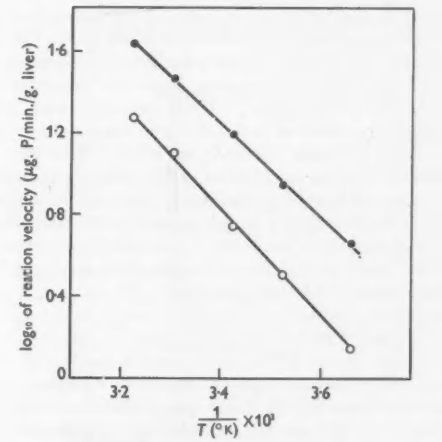

Fig. 10. Influence of temperature on free and total activity at pH 6·1. ○, free activity measured on intact granules incubated in 0·05 M glycerophosphate, pH 6·1, and 0·25 M sucrose; ● total activity, measured similarly on blender-treated granules.

Influence of pH. As is shown in Fig. 9, a distinct difference between free and total enzyme becomes evident when the pH is varied. As a result, the proportion of total enzyme estimated as free activity is not constant, but varies from 9% at pH 5 to 14% at pH 6·1 and 23% at pH 6·9. An increase in the rate of destruction of the granules during the assay can hardly account for these results, since a less acid pH value should have a protective effect (Fig. 8). On the other hand, this explanation could account for the slight relative increase in free activity observed in the very acid range.

Influence of temperature. The temperature coefficient of the reaction was measured at pH 6·1 in order to minimize the danger of activation during the enzyme assay. The liberation of inorganic P proceeded linearly with time, even with the suspension of granules, and made it possible to obtain accurate values for the velocity of the reaction at each temperature. These values furnish very satisfactory Arrhenius plots, as shown in Fig. 10. However, the slope of the two lines is not the same. Using the method of least squares, it was calculated that the energy of activation is $10\,300 \pm 250$ cal./mole for the blender-treated preparation, and $11\,800 \pm 250$ cal./mole for the free activity of the intact suspension. The difference between the two values is statistically significant ($P < 0{\cdot}01$).

DISCUSSION

The new data presented here are compatible with the sac-like representation proposed in an earlier paper for the acid phosphatase-containing granules (Berthet *et al.* 1951). However, since it has now been found that these granules are entirely distinct from those which contain cytochrome oxidase (Duve *et al.* 1953; Appelmans *et al.* 1955), the conclusions arrived at cannot any more be taken to apply to the mitochondria in general. Conversely, morphological observations may have no bearing on the properties of the acid phosphatase-containing granules, for the latter are probably much less abundant than the oxidizing type in the usual mitochrondrial fractions. As was pointed out previously, the two approaches lead to similar conclusions with respect to the permeability properties of the 'mitochondrial' membrane. Moreover, examples of structural hindrance, recalling the facts observed with acid phosphatase, have been described for other enzymes, such as glutamic dehydrogenase (Hogeboom & Schneider, 1953), aconitase (Dickman & Speyer, 1954) and succinoxidase (Tyler, 1954), which probably belong to true mitochondria. These facts suggest that the

two types of granules may not be too dissimilar, but the possibility remains that significant differences may show up in other studies. For instance, the reversibility of the water activation of succinoxidase, observed by Tyler (1954), is in contrast with the essential irreversibility of the osmotic release of acid phosphatase, reported in this paper.

Some additional comments are necessary with respect to the interpretation of the data. In the earlier work, a sharp distinction was made between free and bound activity. The former was attributed exclusively to diffusible enzyme, either present in true solution or adsorbed upon the outer surface of particulate material, whilst the enzyme present in intact granules was taken to be entirely inaccessible to its substrate, owing to the impermeability of the membrane to β-glycerophosphate. The fact that no release of enzyme occurred when the granules were kept in 0·25M glycerophosphate was brought forward in support of this contention. In the present work, the osmotic nature of the protection afforded by glycerophosphate has been established in a quantitative manner with small concentrations of substrate, thus strengthening the earlier evidence. However, attention must be called to a possibility which was not taken into account in the previous reasoning.

It is true for any inert substance that it can only afford osmotic protection if it is unable to penetrate through the membrane. Otherwise it will enter together with sufficient water to maintain osmotic equilibrium, and will cause the granules to swell as they do in distilled water, but at a rate limited by the rate of diffusion of the solute. Examples of this phenomenon have been described by Berthet *et al.* (1951) and in the present paper (Figs. 4, 5). However, if the solute which penetrates is altered inside the granules and converted into products for which there exists a negative concentration gradient, causing them to diffuse out of the granules, perfect osmotic protection remains compatible with some degree of permeability, provided the action of the enzyme and the back-diffusion of the products of reaction are able to keep pace with the inward diffusion of the substrate, thus preventing the latter from accumulating within the granules.

Since this could be the case for glycerophosphate, the possible contribution of the enzyme within the particle to the free activity must be re-examined. A consideration of all the data available indicates that this contribution, if it exists at all, must be very small at pH 5. The free activity under these conditions can be less than 10% of the total activity and is due mainly to dissolved and adsorbed phosphatase (Berthet *et al.* 1951), leaving very little for the possible contribution of the internal enzyme. In addition, if the latter were somewhat accessible to external substrate, one would expect its contribution to increase with increasing glycerophosphate concentration. That such is not the case is indicated by the results of Table 1.

On the other hand, a few facts suggest that the inaccessibility between internal enzyme and external substrate may become less complete as the pH increases. At least such a hypothesis could account for the anomalous pH/activity curve of the free activity (Fig. 9) and for the difference in the temperature coefficients of the free and total activities, as measured at pH 6·1 (Fig. 10). It is possible that the bivalent glycerophosphate ion enters the granules more easily than the univalent ion. Of interest in this respect is the finding by Cleland (1952) that isolated sarcosomes are more permeable to the bivalent than to the univalent inorganic phosphate ion. Dickman & Speyer (1954) have recently reported a similar difference in the pH/activity curve of mitochondrial and soluble aconitase, which they also ascribe to selective permeability. In their case, the permeability to *iso*citrate appeared to decrease with increasing pH.

Attention must be called to the peculiar shape of the osmotic activation curves described in this paper. As is shown in Fig. 7, the process is very gradual and the lysis curves, unlike those obtained in studies on erythrocytes (Hunter, 1940), extend practically over the entire range of osmotic pressure below isotonicity. This fact suggests that great differences must exist between individual granules with respect to their resistance to osmotic disruption.

Thermal activation has been ascribed to autolytic changes of the membrane, possibly occurring in an autocatalytic manner (Berthet *et al.* 1951). The effect of pH on this process (Fig. 8) is consistent with the above hypothesis, since the preparation contains a cathepsin, with a strongly acid pH optimum, which is released in the same manner as acid phosphatase (Duve *et al.* 1953). However, if the observation of Cleland (1952) that the permeability of heart sarcosomes to sucrose increases with decreasing pH is applicable to liver granules, the phenomenon could also be osmotic in nature and occur as a consequence of the penetration of sucrose and water within the particles.

SUMMARY

1. The osmotic activation of the bound acid phosphatase of rat liver has been studied as a function of the sucrose concentration of the medium to which the granules are exposed. The lysis curves are S-shaped and extend over the entire range of osmotic pressure below isotonicity, indicating great variability in the resistance of individual granules to osmotic disruption.

2. The partial activation which occurs in hypotonic media takes place very rapidly, does not show a further increase if the time of exposure at 0° is prolonged and is not reversed by a return to isotonicity.

3. Inorganic univalent salts afford only transient osmotic protection. In contrast, part of the sucrose can be replaced in the medium by an iso-osmolar quantity of β-glycerophosphate, with no adverse effect on the stability of the granules. 2:4-Dinitrophenol is without significant influence on the activation of acid phosphatase.

4. The activation which occurs when the granules are incubated at 37° in 0·25 M sucrose is much more rapid at pH 5 than at pH 6·1.

5. The internal enzyme appears to be entirely inaccessible to external glycerophosphate at pH 5, but seems to contribute significantly to the free activity of intact preparations as the pH is raised, or as the temperature is increased at pH 6·1.

6. The results obtained afford additional support for the sac-like representation of the acid phosphatase-containing granules arrived at in earlier work. It is pointed out that a limited degree of permeability of the membrane to glycerophosphate remains compatible with perfect osmotic protection by this compound, as long as its rate of inward diffusion does not exceed the rate at which it can be hydrolysed within the granules and eliminated in the form of glycerol and inorganic phosphate.

These investigations have been supported by grants from the 'Centre National de Recherches sur la Croissance normale et pathologique', the Rockefeller Foundation and the Lilly Research Laboratories.

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Tissue Fractionation Studies

4. COMPARATIVE STUDY OF THE BINDING OF ACID PHOSPHATASE, β-GLUCURONIDASE AND CATHEPSIN BY RAT-LIVER PARTICLES

By R. GIANETTO* and C. de DUVE
Department of Physiological Chemistry, University of Louvain, Belgium

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In a study of the β-glucuronidase of mouse liver, Walker (1952) has described a number of observations which resemble in a striking fashion the findings made in this laboratory on the unspecific acid phosphatase of rat liver (Berthet & Duve, 1951; Berthet, Berthet, Appelmans & Duve, 1951; Appelmans & Duve, 1955). Like acid phosphatase, β-glucuronidase occurs largely in particulate form; it is partly unreactive when in the bound state and is rendered simultaneously soluble and fully active by all the treatments which have been found to cause a similar release of acid phosphatase. After it was verified in preliminary experiments that the findings of Walker (1952) also apply to the β-glucuronidase of rat liver, it was decided to make a parallel study of the release of the two enzymes, using the methods previously worked out for the investigations on acid phosphatase.

In a search for other enzymes of the same type in rat liver, it was found that cathepsin, whose intracellular distribution recalls in some respects that of acid phosphatase (Maver & Greco, 1951), also exhibits the same general pattern of behaviour. Accordingly, the release of this enzyme was also studied in a number of comparative experiments with acid phosphatase. These studies, which have been reported elsewhere in condensed form (Duve, Gianetto, Appelmans & Wattiaux, 1953), have revealed a close parallelism, both qualitative and quantitative, in the manner of release of the three enzymes.

* Post-doctorate fellow of the National Research Council of Canada. Present address: Department of Biochemistry, University of Montreal.

EXPERIMENTAL

Methods

All the experiments were performed on twice-washed mitochondria fractions, isolated in 0·25 M sucrose from the livers of rats fasted for 12 hr., by the method previously described (Appelmans & Duve, 1955). The preparations were subjected to the treatment chosen to cause partial activation, following which the free enzymic activities were determined by incubation for 10 min. at 37° in the presence of the substrate and of sufficient sucrose to make the concentration of this sugar 0·25 M in the mixture. Total activities were measured in a similar manner on preparations previously exposed for 3 min. to the action of the Waring Blendor, usually after a tenfold dilution in distilled water. Care was taken to prevent overheating during the blendor treatment.

Acid phosphatase. This was determined as before, by measuring the amount of inorganic P set free in the presence of 0·05 M β-glycerophosphate and 0·05 M acetate buffer, pH 5. The total volume of the incubation mixture was 2 ml.

β-Glucuronidase. This was assayed by an adaptation of the method of Talalay, Fishman & Huggins (1946) as modified by Kerr & Levvy (1951). The incubation was performed in a total volume of 2 ml., containing 0·00125 M phenolphthalein glucuronide and 0·075 M acetate buffer, pH 5·2. The reaction was stopped by adding 6 ml. of a solution containing 0·133 M glycine, 0·067 M-NaCl and 0·083 M-Na_2CO_3, pH 10·7. The mixture was clarified by filtration or high-speed centrifuging and its phenolphthalein content determined in a Klett-Summerson photoelectric colorimeter, using a green light-filter (no. 54). A blank containing all the ingredients with the exception of phenolphthalein glucuronide was run in an identical manner. The contribution of the substrate itself to the light absorption was estimated separately; it was always very low. The liberation of phenolphthalein, as measured in this way, proceeds linearly with time and is proportional to the amount of enzyme added. Sucrose is without effect on the determination of phenolphthalein but causes a 25–30% inhibition of enzyme activity at 0·25 M concentration. Since all assays were performed in isotonic sucrose, the inhibition was assumed to be constant in all cases and was not taken into account.

Cathepsin. Special difficulties were encountered in an attempt to adapt the cathepsin assay of Anson (1937) to the needs of the present study. In the first place, tests for free activity had to be carried out at pH 5 and for not longer than 10 min., because the granules are too unstable at a lower pH and do not resist longer incubation at 37°. However, the enzyme is only one-quarter as active at pH 5 as at pH 3·6. Since, in addition, the cathepsin activity of rat liver is already fairly weak and often only a fraction of the total enzyme was measured, reliable assays would have required larger amounts of material than was practical in most cases. A second difficulty arose from the necessary presence of large quantities of sucrose. This sugar is progressively hydrolysed in the trichloroacetic acid which is used to stop the reaction, giving rise to hexoses which cause a significant reduction of the Folin–Ciocalteu reagent. Finally, it was found that the relationship between activity and duration of incubation or enzyme concentration, while perfectly linear, does not however start exactly at the origin of the axes. Apparently, a slight addition to the blank occurs during the initial stage of the incubation. The shortest time required to attain linearity was found to be 2 min.

After exploring various possibilities, the following procedure was finally adopted. The incubation mixture contained, in a total volume of 3 ml., 0·00026 M haemoglobin and 0·17 M acetate buffer pH 5, in addition to the enzyme and 0·25 M sucrose. Duplicate samples were incubated at 37° for 2 and 10 min., respectively. The reaction was stopped by adding 5 ml. of ice-cold 0·3 M trichloroacetic acid; the mixture was cooled in ice immediately and filtered in the ice box. Aromatic degradation products were measured on the filtrates by means of the Folin–Ciocalteu reagent, with tyrosine as standard, as in the original method of Anson (1937). The difference between the readings found with the two samples served to calculate the tyrosine equivalent set free in 8 min. When applied carefully, this method furnishes a reliable assay despite the presence of sucrose. However, the fact that the measured activities were usually low rendered a certain degree of inaccuracy inevitable.

Materials

Phenolphthalein glucuronide. This was isolated as the crystalline cinchonidine salt from the urine of rabbits injected with phenolphthalein phosphate, according to a modification of the method of Talalay *et al.* (1946), supplied to us by the Rowett Research Institute, Bucksburn, Aberdeenshire. The recrystallized product contained neither inorganic phosphate nor free phenolphthalein and was found to yield the theoretical amount of phenolphthalein after acid hydrolysis. The substrate was prepared by extracting the free acid with ethyl acetate, evaporating to dryness, and redissolving in water together with enough NaOH to bring the pH to 5·2.

Crystalline haemoglobin. This was prepared from washed ox erythrocytes, by a method involving dialysis against saturated $(NH_4)_2SO_4$. The crystals were redissolved in water and dialysed exhaustively against distilled water. The concentration of the resulting solution was measured by the reduced pyridine haemochromogen method (Duve, 1948).

Protein. This was determined by the technique of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

As will be obvious from the results reported below, the bound forms of β-glucuronidase and cathepsin share the main properties described previously for particulate acid phosphatase; largely unreactive towards their respective substrates under the conditions of the free activity assays, they are both released in soluble form and activated 5- to 10-fold by the treatments which liberate acid phosphatase. Once these points were established, it seemed of interest to ascertain to what extent the observed similarities hold true on a quantitative basis. Advantage was taken for this purpose of the various means known to elicit a partial and graded activation of acid phosphatase. The release of the other two hydrolases was studied under the same conditions, in experiments which always included simultaneous measurements of acid phosphatase, to

serve as a basis of comparison. For practical reasons, a greater number of experiments were made on β-glucuronidase than on cathepsin.

Mechanical disruption

By running the Waring Blendor at 130 v instead of 220 v, it was possible to activate preparations in a graded manner by means of this instrument. Fig. 1 shows the results of two experiments in which parallel measurements of free activity were made after increasing times of exposure to the Waring Blendor. It is seen that the release of β-glucuronidase and cathepsin both follow that of acid phosphatase very closely.

Activation by freezing and thawing

As illustrated in Fig. 2, a similar parallelism obtains when repeated freezing and thawing is used to activate the preparations.

Osmotic activation

The method used to study the osmotic activation of the three enzymes was based on the experiments of Appelmans & Duve (1955). Granules were exposed for 15 min. at 0° to media of decreasing sucrose concentration, following which samples of each dilution were added to a substrate mixture containing enough sucrose to make the final concentration of this sugar 0·25 M in the enzyme assay. The results of two experiments of this type are shown in Figs. 3 and 4.

Activation by salts

As shown in earlier experiments (Berthet *et al.* 1951; Appelmans & Duve, 1955), the acid phosphatase-containing granules do not retain their integrity when kept at 0° in isotonic NaCl or KCl, whereas they remain perfectly stable under the same conditions in 0·25 M sucrose. The data in Fig. 5 indicate that the same is true for bound β-glucuronidase. In this experiment, the free acid phosphatase and β-glucuronidase activities were measured at regular intervals on preparations kept at 0° in 0·25 M sucrose and in 0·15 M-NaCl, respectively. Following the directions of Appelmans & Duve (1955), the substrates added to each sample contained enough sucrose and NaCl to make the respective concentrations of these substances 0·25 M and 0·15 M in all cases, during the enzyme assay.

Thermal activation

Fig. 6 summarizes the results of three experiments, in which the preparation was activated in 0·25 M sucrose by incubation at 37° and pH 5. Since the three experiments gave almost identical results, only the mean values are given.

Chemical separation

Once released, acid phosphatase and β-glucuronidase behave as distinct soluble proteins. To verify this fact a soluble extract was prepared by

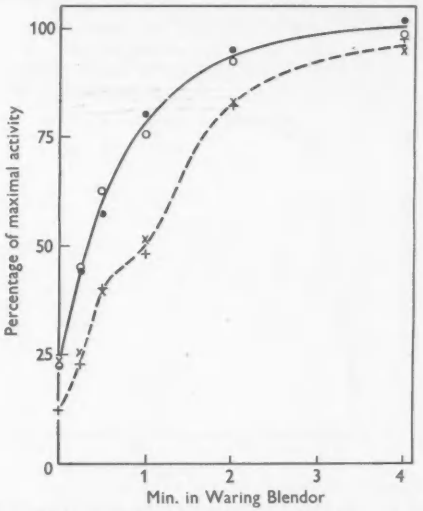

Fig. 1. Activation of acid phosphatase and β-glucuronidase or cathepsin by disintegration of particles in a Waring Blendor. Preparations diluted in 0·25 M sucrose and treated in a Waring Blendor running at low speed (130 v instead of 220 v) for increasing periods of time. Expt. 1, acid phosphatase (●) and β-glucuronidase (○); Expt. 2; acid phosphatase (×) and cathepsin (+).

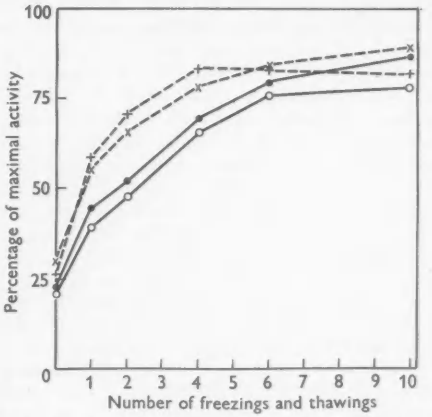

Fig. 2. Activation of acid phosphatase and β-glucuronidase or cathepsin by repeated freezing and thawing. Preparations diluted in 0·25 M sucrose and frozen and thawed an increasing number of times. Expt. 1; acid phosphatase (●) and β-glucuronidase (○); Expt. 2; acid phosphatase (×) and cathepsin (+).

treating a suspension of granules in the Waring Blendor in the presence of 0·1 M acetate buffer, pH 5, incubating the mixture 30 min. at 37° and centrifuging at high speed. The supernatant was dialysed against distilled water, clarified by centrifuging and fractionated with ammonium sulphate in the presence of 0·1 M acetate buffer, pH 5. Each precipitate was collected, dialysed free from ammonium sulphate and analysed for acid phosphatase, β-glucuronidase and protein. The specific activities of each fraction are summarized graphically in Fig. 7.

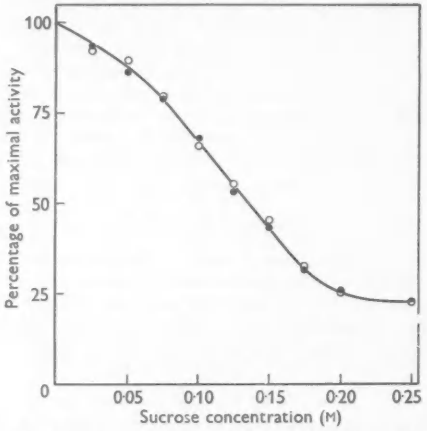

Fig. 3. Activation of acid phosphatase and β-glucuronidase in hypotonic media. Preparations exposed for 15 min. at 0° to sucrose concentration indicated on abscissa. Enzyme assays carried out in 0·25 M sucrose. ●, acid phosphatase; ○, β-glucuronidase.

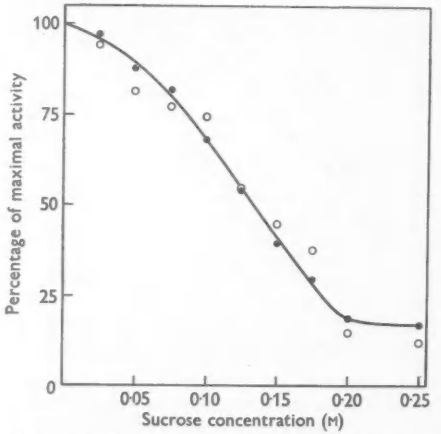

Fig. 4. Activation of acid phosphatase and cathepsin in hypotonic media. Experimental conditions as in Fig. 3. ●, acid phosphatase; ○, cathepsin.

The method outlined above can provide a useful starting-point for the purification of acid phosphatase. In this case the blender extract is incubated at 50°, resulting in a greater denaturation of inactive proteins. By collecting the fraction precipitating between 40 and 75% saturation of ammonium sulphate, and refractionating twice between 58 and 67% saturation, a 300-fold purification over the original tissue is easily achieved.

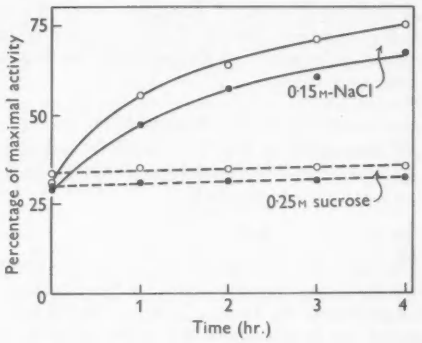

Fig. 5. Activation of acid phosphatase and β-glucuronidase in 0·15 M-NaCl. Preincubation at 0° in 0·25 M sucrose or in 0·025 M sucrose and 0·15 M-NaCl. All enzyme assays carried out in 0·25 M sucrose and 0·15 M-NaCl. ●, acid phosphatase; ○, β-glucuronidase.

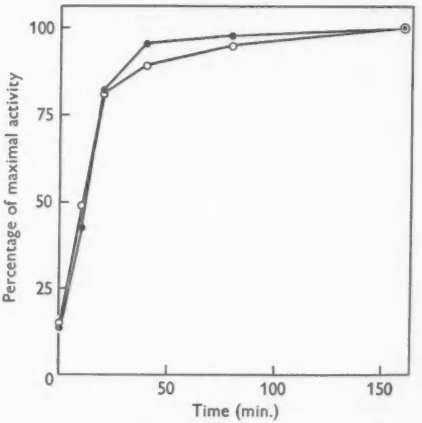

Fig. 6. Thermal activation at pH 5 of acid phosphatase and β-glucuronidase. Preincubation at 37° in 0·25 M sucrose and acetate buffer, pH 5. Mean of three experiments: 1 and 2 in 0·01 M acetate buffer; 3, same preparation as in 2, but in 0·1 M acetate buffer. Acid phosphatase and β-glucuronidase both assayed at pH 5. The values at 169 min. are taken as 100% activity. ●, acid phosphatase; ○, β-glucuronidase.

DISCUSSION

The present investigations were prompted largely by the similarities which were noted between the results of Walker (1952) on the β-glucuronidase of mouse liver and the observations made in this laboratory (Berthet & Duve, 1951; Berthet *et al.* 1951; Appelmans & Duve, 1955) on the acid phosphatase of rat liver. These similarities become even more striking when the two enzymes are studied simultaneously on the same material by means of identical techniques. In addition, they have been found to extend also to cathepsin, another hydrolase with an acid pH optimum, for which this type of behaviour had not been described before.

In the case of bound acid phosphatase, previous investigations have shown that its properties can be entirely explained by the assumption that the enzyme is retained within the granules by a membrane-like barrier showing a low degree of permeability towards β-glycerophosphate. Even though the evidence available for the other two hydrolases is somewhat less complete, it seems fairly safe to conclude, by analogy, that they are likewise enclosed within sac-like granules, surrounded by a membrane impermeable to their respective substrates.

In examining the data on a quantitative basis, one is struck by the close correlations which have been found in the greatest number of experiments. Slight discrepancies have been observed occasionally, but their significance is doubtful in view of the numerous causes of error which are likely to affect experiments of this type. Irregularities, such as occur in some cathepsin experiments, are easily accounted for by the difficulties which complicate the measurements of free activity with this enzyme. These difficulties have been stressed in the section on methods. On the other hand, the cases in which one of the curves appears to be shifted somewhat with respect to the other could arise either from unequal contaminations of the granules by adsorbed enzyme or from errors in the estimation of the total activities, due for instance to a slight denaturation of one of the enzymes in the course of the blender treatment. This interpretation is supported by the fact that even the initial free activities differ in the two experiments in which such shifts have been observed (β-glucuronidase experiment of Figs. 2 and 5).

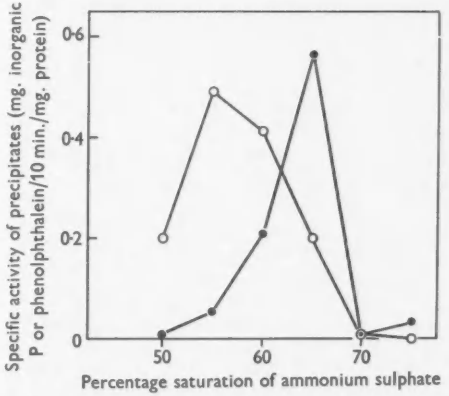

Fig. 7. Separation of acid phosphatase and β-glucuronidase by ammonium sulphate fractionation. Specific activities of fractions precipitated from a soluble extract of large granules at increasing ammonium sulphate concentrations. ●, acid phosphatase; ○, β-glucuronidase.

The various causes of error referred to above could mask dissociations as well as correlations, and it is therefore fair to state that the evidence produced is compatible with the view either that the three enzymes are released in an identical manner in all the circumstances investigated or that the processes are only grossly similar and distinguished by slight but significant differences. It seems, however, justified to attach more weight to the experiments in which almost perfect correlations were observed, since they are more numerous and offer no indication, as do the others, of having been complicated by an artifact.

Turning now to the actual interpretation of the results, it should be pointed out that the observed analogies require a great number of similarities to exist between the granules containing the three enzymes, for the particular shape of an activation curve depends on a whole set of factors, which are not the same for each activating agent used. For instance, the osmotic activation curve reflects the statistical distribution of a group of properties which determine what is usually termed 'membrane resistance'. To these properties must be added the permeability of the membrane to NaCl, to account for the activation which obtains in salt solutions. On the other hand, an instrument such as the Waring Blendor may be expected to hit in a much more random manner, although even here some properties of the granules may be involved. Again, the susceptibility to freezing and thawing, pH or temperature must be dependent to a certain extent on inherent characteristics of the granules.

Barring the possibility of a chemical link between the three enzymes—a hypothesis which has been disproved experimentally, at least for acid phosphatase and β-glucuronidase—the most obvious conclusion is that they are actually associated within the same granules. This view, which has been put forward in a previous report (Duve *et al.* 1953), was rendered all the more likely by the finding, in preliminary experiments, that the distribution pattern of the two other hydrolases is similar to that

found for the acid phosphatase-containing granules. As will be shown in a subsequent paper (Appelmans, Wattiaux & Duve, 1955), these granules form a special group, entirely distinct from the oxidizing mitochondria and from the microsomes, and are probably represented by a relatively small number of individuals. Their cytological nature is unknown and any new information which could be obtained regarding their structure and enzymic equipment appeared therefore as particularly valuable. These facts have been commented upon also in a recent review (Duve & Berthet, 1954).

Experiments now in progress have thrown some doubt on the above interpretation, showing that the distribution of β-glucuronidase is not identical with that of acid phosphatase. The difference is slight, involving a shift of about 15% of β-glucuronidase from a lighter mitochondrial fraction to the microsomes, but it is reproducible and clearly beyond recognized experimental errors. Studies on tumours and precancerous tissues have also furnished indications that the two enzymes may behave in an independent manner. These investigations, which will be reported in later publications, must be pursued further before allowing definite conclusions. But they are mentioned in view of the important incidence they may have on the final interpretation of the results presented here.

SUMMARY

1. The β-glucuronidase and cathepsin of rat liver share a number of the properties previously described for acid phosphatase. Like this enzyme, they are enclosed within cytoplasmic granules and exert their full activity in an *in vitro* assay only if the granules have been subjected to a treatment which simultaneously releases the enzymes in soluble form.

2. The release of the three enzymes proceeds almost identically in washed particulate preparations activated either by the Waring Blendor, freezing and thawing or exposure to hypotonic media. Incubation at 0° in 0·15M-NaCl, or at 37° and pH 5 in 0·25M sucrose also causes a parallel liberation of β-glucuronidase and acid phosphatase. Cathepsin was not measured in these experiments.

3. Once released, acid phosphatase and β-glucuronidase are easily separable by means of ammonium sulphate fractionation. The partial purification of acid phosphatase is described.

4. In discussing the results obtained, it is concluded that the three enzymes behave essentially as though they were associated within the same granules. However, recent unpublished data suggest that this conclusion may have to be revised.

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Tissue Fractionation Studies

5. THE ASSOCIATION OF ACID PHOSPHATASE WITH A SPECIAL CLASS OF CYTOPLASMIC GRANULES IN RAT LIVER

BY FRANÇOISE APPELMANS, R. WATTIAUX AND C. DE DUVE
Department of Physiological Chemistry, University of Louvain, Belgium

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In a previous study of the intracellular distribution of acid phosphatase in rat liver, it was found that the bound form of the enzyme, although associated mainly with the mitochondria, is also recovered to some extent with the microsomes (Berthet & Duve, 1951). Similar observations were made by Palade (1951). Other mitochondrial enzymes, such as cytochrome oxidase, do not show this peculiarity, and it seemed of interest to ascertain the cause of the discrepancy. The investigations which were undertaken in order to clarify this point are reported in the present paper. They have led to the discovery that acid phosphatase is attached to a special type of cytoplasmic granules, differing both from the cytochrome oxidase-bearing mitochondria and from the glucose 6-phosphatase-containing micro-

somes. A new fractionation scheme has been developed as a result of these findings. Preliminary reports of these results have appeared elsewhere (Duve, Appelmans & Wattiaux, 1952; Duve, Gianetto, Appelmans & Wattiaux, 1953).

METHODS

Differential centrifuging

In most experiments, the starting material was a 0·25 M sucrose cytoplasmic extract, free of nuclei, prepared from the livers of rats fasted for 12 hr., by the technique described previously (Berthet & Duve, 1951; Appelmans & Duve, 1955).

The methods used for separating particulate fractions from this material were varied extensively and will be described in the text. To ensure reproducibility, the conditions of centrifuging will be defined in each case by the time-integral of the field prevailing in the middle of the fluid, expressed in the composite unit *g*-min., as well as by the sedimentation constant $S_{min.}$ of the lightest spherical particles to be sedimented completely, expressed in Svedberg units ($1S = 10^{-13}$ sec.). This nomenclature has been justified elsewhere (Duve & Berthet, 1953). The characteristics of the centrifuges used are given in Table 1. Attention is called to the last column of this table, showing the conditions required in each centrifuge to sediment completely the same group of particles, taking as standard of comparison the no. 40 rotor of the Spinco centrifuge. These values illustrate the statement that 'individual centrifuges... are less comparable, so far as complete sedimentation is concerned, when developing equal average fields than when turning at equal velocities' (Duve & Berthet, 1953).

Separation of fractions was performed by means of a pipette with a narrow drawn-out tip bent to an angle of 90°, operated with a rubber bulb fitted with valves, allowing controlled sucking. In low-speed centrifuging, this step becomes the most variable factor, as part of the sediment is loosely packed and easily removed with the supernatant fluid. The sediments were resuspended by means of a micro-homogenizer of the Potter & Elvehjem (1936) type.

Enzyme assays

Acid phosphatase. This was measured as before at 37° in the presence of 0·05 M β-glycerophosphate and 0·05 M acetate, adjusted to pH 5 with HCl. For measurements of free activity, the incubations were carried out for 10 min. in the presence of 0·25 M sucrose, under which conditions the enzyme enclosed within intact granules is usually unreactive (Appelmans & Duve, 1955). Total activity was estimated on preparations previously treated 3 min. in the Waring Blendor, usually after a 1:10 dilution with distilled water, or frozen and thawed at least 10 times. Bound activity is obtained as the difference between total and free activity.

Glucose 6-phosphatase. This was determined by the amount of inorganic phosphate formed at 37° in the presence of 0·008 M glucose 6-phosphate and 0·05 M cacodylate buffer, pH 6·5. The substrate was prepared by an enzymic method (Hers, Beaufays & Duve, 1953).

Cytochrome oxidase. This was assayed by the optical test of Cooperstein & Lazarow (1951), which was run in the Beckman Model DU spectrophotometer, using cells of 1 cm. light path. A suitable dilution of the enzyme was first prepared in 0·005 M phosphate buffer, pH 7·4, and kept for a few minutes at 0°. This precaution was necessary to obtain maximal initial rates. The reaction was started by adding 0·1 ml. of this dilution to 3 ml. of a solution containing $1·7 \times 10^{-5}$ M reduced cytochrome *c* in 0·03 M potassium phosphate buffer, pH 7·4. The decrease in optical density at 550 mμ. was measured at regular intervals for 3–6 min., following which the blank was read after addition of a small amount of solid potassium ferricyanide to the mixture.

Under these conditions the oxidation of cytochrome *c* is practically of first order with respect to the concentration of reduced pigment. One unit of activity is defined as the amount of enzyme causing the decadic logarithm of the concentration of reduced cytochrome *c* to decrease by one unit/min./100 ml. of incubation mixture. The calculations are performed as described by Cooperstein & Lazarow (1951). The cytochrome *c* was prepared from ox or horse heart by the method of Keilin & Hartree (1945). Before assay, enough $Na_2S_2O_4$ was added to reduce approximately 90% of the cytochrome. The rate of autoxidation was negligible.

The medium used in the measurements of O_2 consumption contained 0·008 M sodium glutamate, 0·008 M sodium fumarate, 0·001 M potassium adenosine triphosphate, 0·001 M-$MgCl_2$, 0·02 M potassium phosphate buffer, pH 7·4, and 0·125 M sucrose. Vessels, containing 2·5 ml. of this mixture and 0·2 ml. of enzyme, were incubated at 30° in a Warburg apparatus, with air as gas phase and 20% (w/v) KOH in the centre well. Readings were taken every 5 min., after an equilibration period of 10 min. The O_2 uptake proceeded linearly during at least 30 min. and the slope of the line was used to calculate the q_{O_2} (μl. O_2/min./g. liver or mg. N).

Nitrogen was estimated by a micro-Kjeldahl method.

RESULTS

Sedimentation experiments

In order to investigate the cause of the atypical distribution found for bound acid phosphatase in previous experiments, the sedimentation behaviour

Table 1. *Characteristics of centrifuges*

Centrifuge	Fluid volume (ml.)	$r_{max.}$ (cm.)	$r_{min.}$ (cm.)	Equivalent relative values*	
				g (av.)†	rev./min.
Corda, type 2047	50	15	8	2·16	1·1
M.S.E., superspeed attachment	7·5	7	3	1·26	1·28
Spinco, model L rotor no. 20	100	12·8	7·5	1·6	1·01
Spinco, model L rotor no. 40	10	8·1	4·8	1	1

* Necessary for the complete sedimentation of the same group of particles (same value of $S_{min.}$).
† Field in the middle of centrifuged fluid, i.e. at 0·5 ($r_{max.} + r_{min.}$) cm. from axis.

of this enzyme was compared with that of the respiratory activity, which has been shown by numerous workers to belong exclusively to mitochondria. At first, the rate of oxygen consumption in the presence of suitable substrates was used to measure respiratory activity. In later investigations, this test was replaced by the simpler cytochrome oxidase assays.

Fig. 1 shows the results of an experiment in which equal amounts of unwashed mitochondria were subjected to increasing centrifugal forces, in the M.S.E. superspeed attachment. The sediments were collected and analysed for nitrogen, free and total acid phosphatase, and respiratory activity. The results obtained, expressed as percentage of the corresponding values for the original suspension, are plotted against the time-integral of the average field applied.

It is seen that the respiratory activity of the sediments increases almost linearly with the centrifugal force and reaches the value of the original suspension at about 50000 *g*-min. ($S_{min.} = 14000\ S$). The sedimentation of bound acid phosphatase is only about half complete at this point, and continues to rise with increasing centrifugal force, but less steeply. Only an insignificant increase of the nitrogen content of the sediments is associated with this second part of the curve.

Similar experiments, in which cytochrome oxidase was measured instead of oxygen consumption, gave essentially the same results. In some cases, anomalous results were obtained. In Fig. 2 are shown sedimentation curves determined on two cytoplasmic extracts prepared from the same liver in 0·25 M and 0·88 M sucrose, respectively. The abnormal shape of the phosphatase curve probably reflects irregular decantation. This step is not easily performed in a reproducible manner when the tubes contain loosely packed precipitates and incompletely sedimented material. The data, however, serve to show that the sucrose concentration of the suspending medium does not materially affect the dissociation between the two activities; this dissociation was observed even in 1·4 M sucrose.

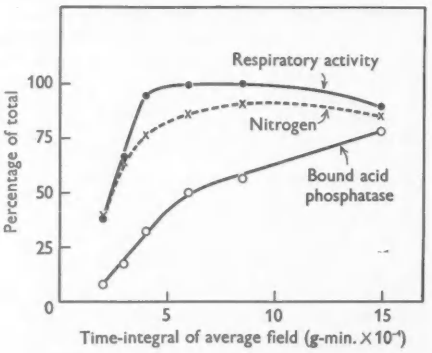

Fig. 1. Sedimentation curves of nitrogen, respiratory activity and bound acid phosphatase of a mitochondrial suspension. Original preparation: unwashed mitochondria, isolated in the M.S.E. superspeed attachment by 200000 *g*-min. ($S_{min.} = 3600\ S$) and diluted 1:10 in 0·25 M sucrose. From this suspension, 7·5 ml. portions were centrifuged at increasing average field-time values (see abscissa) in the M.S.E. centrifuge. The sediments were collected and analysed. The results are expressed as percentage of the total amounts present in the same volume of the original suspension.

Separation experiments

In a number of experiments, it could be shown that the partial dissociation of acid phosphatase from respiration, brought about by a single sedimentation, is further increased by the use of procedures known to favour the separation of particles of differing sedimentation constant.

One series of experiments, aimed at isolating a head fraction in as great a state of purity as possible, was performed as follows. Starting from a 1:10 cytoplasmic extract

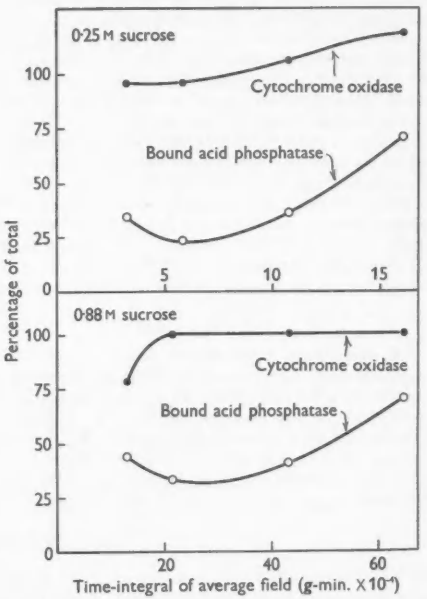

Fig. 2. Influence of sucrose concentration on dissociation between bound acid phosphatase and cytochrome oxidase. Sediments isolated from 10 ml. portions of 1:14 cytoplasmic extracts, in no. 40 rotor of Spinco centrifuge. Upper half: extract prepared in 0·25 M sucrose; lower half: extract prepared in 0·88 M sucrose from the same liver. For significance of coordinates, see Fig. 1.

in 0·25 M sucrose, the heavier granules, termed 'head fraction', were separated in the Corda centrifuge by 35 000 *g*-min. ($S_{min.} = 35\,000\ S$) and washed twice under the same conditions. A fairly large amount of loosely packed particles were removed, together with the first supernatant, which was spun in the M.S.E. superspeed attachment at 120 000 *g*-min. ($S_{min.} = 6000\ S$). The resulting sediment was resuspended in the first washing of the head fraction and recentrifuged at the same speed. The second washing of the head fraction served to wash the new sediment in the same manner, yielding the lighter 'main fraction'. Three experiments of this type were performed. In a fourth experiment, a 'head fraction' was isolated as before, but a combined 'head + main fraction' was prepared directly in the M.S.E. centrifuge from a separate portion of the cytoplasmic extract.

The two fractions were subjected to various analyses, the results of which are summarized in Table 2. To facilitate comparison between experiments, 'main' values were calculated in the fourth experiment by substracting the 'head' values from those obtained on the combined 'head + main' fraction.

The heavier 'head' fraction, isolated by this procedure, contained one-third to two-fifths of the total mitochondrial nitrogen and of the respiratory activity. The remainder was recovered in the 'main' fraction. In general appearance, the two fractions were almost undistinguishable, except for a slightly pinker tinge in the 'main' fraction, indicating a greater contamination by microsomes, also reflected in the glucose 6-phosphatase assays. Microscopic examination showed a greater proportion of smaller granules in the 'main' fraction but no other obvious difference.

In contrast, striking differences were found in the acid phosphatase content of the two fractions. The specific activity of this enzyme in total homogenates usually lies between 50 and 70 μg. P/10 min./mg. N. It is consistently lower in the 'head' fraction and much higher in the 'main' fraction. This difference becomes still more apparent if the bound activities are considered. In Expt. 3, particularly, bound phosphatase was almost absent from the 'head' fraction and 30 times more abundant in the 'main' fraction. Here again, the unequal distribution of acid phosphatase is not reflected in the partition of nitrogen, since the specific q_{O_2} of the two fractions is practically the same.

Table 3 summarizes the results of an experiment in which the layering technique, first introduced by Hogeboom, Schneider & Palade (1948), was used. This experiment was carried out in the no. 20 rotor of the Spinco centrifuge, accommodating tubes of 100 ml. capacity. Ten ml. of a 1:6 cytoplasmic extract in 0·25 M sucrose were first pipetted into the tube, following which 90 ml. of 0·3 M sucrose were slowly introduced through this layer by means of a thin capillary, the tip of which extended to the bottom of the tube. As a result, the extract was layered over the sucrose, with a fairly sharp interface. After centrifuging at 65 000 *g*-min. ($S_{min.} = 14\,000\ S$), the supernatant was decanted and centrifuged at 225 000 *g*-min. ($S_{min.} = 4000\ S$). The two sediments and the final supernatant were assayed for cytochrome oxidase and bound acid phosphatase.

As is shown in Table 3, the layering procedure which should, on theoretical grounds, allow a more efficient separation of particles of different sedimentation constant, does in fact emphasize the dissociation between cytochrome oxidase and bound acid phosphatase. So does repeated washing, as comes out clearly in the balance presented in Table 4.

The starting material, in this experiment, was a 1:6 cytoplasmic extract in 0·25 M sucrose. A heavier sediment was separated in the no. 40 rotor of the Spinco centrifuge by 54 000 *g*-min. ($S_{min.} = 10\,500\ S$) and washed twice at

Table 2. *Subfractionation of mitochondria*

The terms 'Head' and 'Main' refer to the two subfractions; for details of experimental procedure, see text.

	Expt. 1		Expt. 2		Expt. 3		Expt. 4	
	'Head'	'Main'	'Head'	'Main'	'Head'	'Main'	'Head'	'Main'*
Nitrogen (mg./g. liver)	2·08	5·16	2·40	5·00	2·40	4·60	2·94	3·76
Acid phosphatase activity (μg. P/10 min./g. liver)								
Total	88	1030	109	620	46	780	103	997
Free	34	304	23	170	26	175	19	53
Bound	54	726	86	450	20	605	84	944
(μg. P/10 min./mg. N)								
Total	42	200	46	124	19	170	35	265
Free	16	59	10	34	11	39	6	14
Bound	26	141	36	90	8	131	29	251
Respiration								
(μl. O_2/min./g. liver)	—	—	—	—	10·4	21·2	15·7	20·7
(μl. O_2/min./mg. N)	—	—	—	—	4·33	4·6	5·35	5·5
Glucose 6-phosphatase (μg. P/10 min./g. liver)	41·2	505·8	—	—	—	—	—	—

* 'Main' values calculated by substracting 'Head' from 'Head + Main' values.

33 000 *g*-min. ($S_{min.}$ = 17 300 *S*). The three supernatants were combined and centrifuged at 160 000 *g*-min. ($S_{min.}$ = 3500 *S*) to separate a lighter sediment. In a parallel experiment, a heavy fraction was isolated in the same rotor by layering 3 ml. of the extract over 6·5 ml. of 0·3 M sucrose and centrifuging at 74 000 *g*-min. ($S_{min.}$ = 7700 *S*).

Despite a fairly large loss of bound phosphatase, owing to injuries suffered by the granules in the course of the experiment, the results of the assays demonstrate the efficiency of washing in separating the two activities. As can be seen in Table 4, each washing removes more bound phosphatase than cytochrome oxidase, yielding a final sediment with a phosphatase:oxidase ratio of 0·12 and a supernatant, from which a lighter sediment is isolated, in which this ratio is 15 times larger. The layering technique gives results comparable to those obtained with one centrifuging and one washing.

Quantitative fractionation

The importance of the dissociation which could be brought about experimentally between two enzymes, showing roughly similar distributions by the conventional fractionation procedure, made it imperative to devise a scheme which would make differences of this kind immediately apparent. On the basis of the results obtained, the following procedure was decided upon.

The nuclei and cytoplasmic extract were separated in the usual manner. The Spinco no. 40 rotor was used for all the subsequent steps of the fractionation. A heavy mitochondrial fraction was first isolated from the cytoplasmic extract by centrifuging at 33 000 *g*-min. ($S_{min.}$ = 17 300 *S*) and washed twice under the same conditions. The combined supernatants were centrifuged at 250 000 *g*-min. ($S_{min.}$ = 2300 *S*) to separate a light mitochondrial fraction, which was also washed twice. The microsomes were sedimented from the resulting supernatants by 3 000 000 *g*-min. ($S_{min.}$ = 190 *S*), and washed, leaving the final supernatant.

The results of a representative experiment are given in Table 5. It will be noted that the oxidase and phosphatase are recovered mostly in the two mitochondrial fractions but are distributed very unequally between them. On the other hand, glucose 6-phosphatase is present in low quantities in these fractions, even in the lighter one, and comes

Table 3. *Subfractionation by layering procedure*

For details of experimental procedure, see text.

Fraction	Percentage of activity of extract			Ratio, bound phosphatase: cytochrome oxidase
	Cytochrome oxidase	Total acid phosphatase	Bound acid phosphatase*	
Cytoplasmic extract	100	100	100 (73)	1
Heavy sediment	88	30	34 (25)	0·39
Light sediment	14	41	51 (37)	3·6
Final supernatant	1·5	26	16 (12)	10·7
Recovery	103·5	97	101 (74)	0·98

* Figures in brackets show bound activity as percentage of total activity of extract.

Table 4. *Efficiency of washing and of layering procedure*

For details of experimental procedures, see text.

Fraction	Percentage of activity of extract			Ratio, bound phosphatase: cytochrome oxidase
	Cytochrome oxidase	Total acid phosphatase	Bound acid phosphatase*	
Cytoplasmic extract	100	100	100 (81)	1
Heavy fraction (H.F.)	88	45	48 (39)	0·55
Supernatant	22	56	49·5 (40)	2·25
H.F. washed once	91	27	25 (20)	0·27
First washing	0	14	13·5 (11)	2·25
H.F. washed twice	73	14	8·5 (7)	0·12
Second washing	5	8	8·5 (7)	1·7
Light fraction, unwashed	26	46	48 (39)	1·85
Final supernatant	3	24	8·5 (7)	2·83
Recovery	102	84	65 (53)	0·64
Layering experiment				
Sediment	82	29	26 (21)	0·32
Supernatant	31	56	42 (34)	1·35
Recovery	113	85	68 (55)	0·60

* Figures in brackets show bound activity as percentage of total activity of extract.

Table 5. *Quantitative fractionation*

For details of experimental procedure, see text.

Fraction	Nitrogen (mg./g. liver)	Percentage of activity of homogenate: Cytochrome oxidase	Total acid phosphatase	Bound acid phosphatase*	Glucose 6-phosphatase
Homogenate†	34·2	100	100	100 (78·5)	100
Nuclei, washed twice	5·3	16·5	7·3	6·2 (4·9)	10·7
Heavy mitochondrial fraction, washed twice	3·9	60·5	20·6	17·6 (13·8)	1
Light mitochondrial fraction, washed once	3·3	20	43·9	47·2 (37)	6·4
Microsomes, unwashed	9·8	2	17·6	11 (8·6)	79·3
Final supernatant	11·3	0	9·2	0	2·3
Recovery	33·6	99	98·6	82 (64·3)	99·7

* Figures in brackets show bound activity as percentage of total activity of homogenate.
† Sum of nuclei + cytoplasmic extract.

Table 6. *Separation of the 'fluffy layer'*

Mitochondria, together with the 'fluffy layer', were spun down in the no. 40 rotor of the Spinco centrifuge by 160 000 *g*-min. ($S_{min.} = 3500\ S$) and washed once at 100 000 *g*-min. ($S_{min.} = 5700\ S$). The top layer of this sediment was removed by means of a glass rod, resuspended and centrifuged at 350 000 *g*-min. ($S_{min.} = 1600\ S$), yielding a well packed sediment, which could easily be separated mechanically into two fractions, containing the pink 'fluffy layer' and the underlying yellow mitochondrial precipitate, respectively. Enzyme activity expressed as μg. P/10 min./g. liver.

Fraction	Acid phosphatase: Activity	Acid phosphatase: %*	Glucose 6-phosphatase: Activity	Glucose 6-phosphatase: %*
Yellow sediment	110	7·5	238	7
Pink 'fluffy layer'	21	1·5	336	10

* Percentage of total activity of extract.

down predominantly with the microsomes. A comparison of these observations with those obtained by the classical technique (Berthet & Duve, 1951) indicates that the main result achieved by the new procedure is a subfractionation of the mitochondria into a heavier fraction, rich in oxidase and poor in phosphatase, and a lighter one, showing an inverse relationship with respect to its content of the two enzymes.

The 'fluffy layer'

The peculiar sedimentation properties of acid phosphatase suggested the possibility that this enzyme might be associated with the pinkish, poorly sedimented 'fluffy layer', which is found on top of mitochondrial precipitates. In the fractionation scheme described above, this layer forms a conspicuous part of the first sediment obtained in the isolation of the light mitochondrial fraction. However, it is usually removed to a large extent in the course of washing and comes down mainly with the microsomes. Therefore the possibility considered seemed rather unlikely. Further evidence against it is furnished by the results of Table 6, showing that the 'fluffy layer' is distinctly poorer in acid phosphatase, but richer in glucose 6-phosphatase, than the lighter portion of the yellow mitochondrial sediment.

DISCUSSION

The results reported are in complete agreement with those of Novikoff, Podber, Ryan & Noe (1953), who have described a partial dissociation of acid phosphatase from succinoxidase, together with certain other data indicative of biochemical heterogeneity in particulate fractions. In assessing the significance of such results, the possibility of artifacts must however be examined. Cytochrome oxidase and the related succinoxidase are strongly attached to the insoluble framework of the mitochondria. Acid phosphatase, on the other hand, is easily released in soluble form from injured granules, and may subsequently undergo an artificial redistribution amongst particulate elements, owing to adsorption phenomena. In the present work, intact granules have been estimated selectively as the difference between total and free activity. The fact that the enzyme recovered in the lighter fractions is present mainly in bound form shows clearly that the separation of acid phosphatase from cytochrome oxidase reflects a true difference in the sedimentation characteristics of the granules in which the two enzymes are concentrated.

The observed heterogeneity could be a property either of the granules themselves or of the isolated fractions. In the first interpretation, which is apparently favoured by Novikoff *et al.* (1953), both enzymes are taken to be present in all the granules, but in a ratio which varies with one of the factors, size or density, which determine the sedimentation

behaviour of the particles. In the other, the fractions are considered as mixtures of two distinct classes of granules, having slightly different sedimentation properties and characterized each by one of the two enzymes studied.

In judging the relative merits of these two hypotheses, it must be kept in mind that fractions isolated by differential centrifuging are always mixtures of granules of differing sedimentation constant. It is theoretically possible to increase the resolution, either by the use of a layering procedure or by repeated washings; but such attempts soon become defeated by the progressive agglutination which occurs in the preparation. As was shown in a previous study (Hers, Berthet, Berthet & Duve, 1951), it is already very difficult to free mitochondria completely from microsomes and one must expect these complications to be even greater with particles of closer sedimentation behaviour.

Examined in this light, the results obtained in the layering and washing experiments stand out as particularly significant. In every instance, these procedures were found to enhance the dissociation between the two enzymes. Under the best conditions, one-third of the respiratory activity was isolated practically free of acid phosphatase, and more than 70 % of the cytochrome oxidase was separated in association with less than 10 % bound phosphatase. Conversely, fractions containing 10–20 % of the original bound phosphatase of the extract and only traces of cytochrome oxidase have also been obtained. As was pointed out above, the nitrogen content of the isolated fractions appeared to correlate essentially with their respiratory activity, irrespectively of their content in acid phosphatase.

Such results suggest very strongly that the classical mitochondrial fraction is made up mostly of granules possessing oxidative activity but devoid of acid phosphatase. In view of the numerous morphological observations made by other authors, these granules can only be the true mitochondria. The results of the sedimentation experiments are in agreement with this conclusion, indicating that the cytochrome oxidase-containing granules behave as a fairly homogeneous population, with a sedimentation constant of approximately 14 000 Svedberg units (in 0·25 M sucrose at 0°); using the limit values assigned by Holter, Ottesen & Weber (1953) to the density of mitochondria (1·10–1·20), this figure would correspond to diameters ranging between 0·55 and 0·94 μ.

Consequently, the main information provided by the present study is that typical mitochondria are probably free of acid phosphatase, but that, when isolated by differential centrifuging, they are contaminated by granules of a second type which are very rich in this enzyme. The cytological identification of this second group of granules raises a difficult problem. In the first place, judging from the sedimentation data, they appear to form a much more heterogeneous population, with sedimentation constants of about 1000–10 000 Svedberg units, making it almost impossible to isolate them in good yield without significant contamination by mitochondria or microsomes. Moreover, the present indications are that they must be very few in proportion. In several experiments, fractions showing an almost tenfold increase in specific phosphatase activity over that of the original homogenate have been isolated. These fractions still contained significant amounts of cytochrome oxidase and glucose 6-phosphatase and it could be estimated that the pure granules must have a specific activity of at least 1500 μg. P/10 min./mg. N and be associated with 4 % or less of the cell's nitrogen. Fractions showing specific activities of this order of magnitude have actually been separated in preliminary trials performed by means of density-gradient centrifuging, and it is hoped that improved methods will make it possible to prepare samples of granules pure enough for morphological examination.

In the meantime, some indirect information is provided by the results recorded in this and previous papers. From the sedimentation data, it can be calculated that the diameters of the granules must range mostly between 0·25 and 0·8 μ. if their density is low (1·10) or between 0·13 and 0·4 μ. if their density is high (1·30). Despite the uncertainty of these calculations, they tend to show that the acid phosphatase-containing granules are comparable in size to small mitochondria rather than to microsomes. This conclusion is further supported by the fact that they can be separated to a large extent from glucose 6-phosphatase, and by the experiments in which they were shown to be associated with the mitochondrial sediment rather than with the superimposed 'fluffy layer'. A confirmation of this finding is to be found in the work of Laird, Nygaard, Ris & Barton (1953), who made an extensive study of the 'fluffy layer' and found no evidence for a concentration of acid phosphatase in this fraction. Finally, it must be recalled that studies of the release of acid phosphatase have led to the conclusion that the granules behave as typical osmotic systems, surrounded by a semi-permeable membrane.

Whether they represent a specialized form of mitochondria or an entirely distinct cytological entity remains to be established. The most comparable bodies are the 'droplets' recently isolated from kidney tissue by Straus (1954), who found them to contain little succinoxidase but considerable amounts of acid phosphatase. These 'droplets' have been studied histologically by Oliver (1948);

they increase greatly in size after the intraperitoneal injection of egg white and are believed by this author to be concerned with the tubular reabsorption of proteins. They are, however, of much larger size than was estimated for the liver granules (0·5–1·5 μ. in untreated animals, 1–5 μ. in animals injected with egg white) and their specific acid phosphatase activity (185–270 μg. P/10 min./mg. N) is distinctly lower than that found in the best liver fractions and much lower, therefore, than the values estimated for the pure liver granules.

In addition to their theoretical interest, the results obtained entail a conclusion of great practical importance, showing that two enzymes may be both recovered to a large extent in the mitochondrial fraction and still be entirely unrelated cytologically. This view is supported by a number of recent reports, describing evidence of biochemical heterogeneity in isolated mitochondria (Novikoff *et al.* 1953; Laird *et al.* 1953; Holter *et al.* 1953; Kuff & Schneider, 1954; Paigen, 1954). As will be shown in a subsequent publication, the use of the new fractionation method outlined in the present paper provides a partial solution to the problems raised by these findings.

SUMMARY

1. A comparative study has been made of the sedimentation properties of bound acid phosphatase and of cytochrome oxidase or the related respiratory activity, in rat-liver fractions. Acid phosphatase has been found to sediment more slowly than the oxidase.

2. The partial dissociation between the two activities, achieved by a single centrifuging, is further enhanced by the use of a layering procedure or by repeated washings.

3. It is concluded from the results obtained that the classical mitochondrial fraction is made up essentially of typical mitochondria, possessing oxidative properties but devoid of acid phosphatase. The latter enzyme is believed to be associated with a special class of granules, very few in proportion, entirely distinct from microsomes and comparable in size to small mitochondria. In the classical fractionation procedures, these granules are unequally distributed between the mitochondria and the microsomes, occurring mostly in the former fraction.

4. A new scheme of fractionation is described, which will make it possible to distinguish between true mitochondrial enzymes and contaminants of the acid phosphatase type.

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The Extracellular Polysaccharide of *Aerobacter Aerogenes* A3 (S1) (Klebsiella Type 54)

By J. F. WILKINSON, W. F. DUDMAN and G. O. ASPINALL
Department of Bacteriology and Department of Chemistry, University of Edinburgh

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Although it has long been known that organisms of the Aerobacter–Klebsiella group produce very mucoid growths, few attempts have been made to investigate the nature of their capsules and slime. Recently, attention has been drawn to the extracellular polysaccharides of these organisms by the work of Edwards & Fife (1952), who showed that these bacteria exist in a large number of immunologically distinct types. Edwards & Fife identified fifty-seven types, but this has since been increased to nearly 100 (Edmunds, 1954; Henriksen, 1954). These extracellular polysaccharides may occur as loose slime or as distinct capsules and the amount produced varies considerably according to the nature of the growth medium (Duguid & Wilkinson, 1953; Wilkinson, Duguid & Edmunds, 1954). They are also responsible for the distinctive antigenic specificity of strains in the mucoid phase (Wilkinson *et al.* 1954).

Chemical studies on the nature of *Aerobacter aerogenes* extracellular polysaccharides have been carried out on untyped strains. Schardinger (1902*a*, *b*) isolated a galactan from a 6-week broth culture of *Aero. aerogenes* but the product may have been an intracellular polysaccharide. Tomcsik (1927) isolated a polysaccharide by treatment of capsulated cells with potassium hydroxide, and Warren (1950) obtained a slime fraction, but neither author identified the component sugars.

No attempts have been made to determine the influence of different carbon sources on the relative amounts of the component sugars in a heteropolysaccharide. Morgan & Beckwith (1939) cultured a strain of *Escherichia coli* on media containing sucrose, glucose, rhamnose or xylose as the carbon source and showed that the immunological specificity of the strain was unaffected. However, this may only mean that a certain immunologically determinant group is present in the extracellular polysaccharide and the overall composition may have varied. Forsyth & Webley (1949) grew various species of the genus *Bacillus* on media containing sucrose, fructose, glucose, galactose or arabinose, and found that the component sugars of the extracellular polysaccharide remained unaltered although no quantitative determinations were carried out.

In the present study, the composition of the extracellular slime polysaccharide of *Aero. aerogenes* strain A 3 (S 1) (Klebsiella Type 54) has been studied after growth on media containing different carbon sources. The constituent sugars have been identified and the different polysaccharides compared.

EXPERIMENTAL AND RESULTS

Growth of organism

Organism. *Aero. aerogenes* A3 (S1) is a non-capsulate organism producing an abundance of extracellular polysaccharide as a viscous slime. Immunologically it belongs to Type 54 of the Edwards classification, and its origin and biochemical characteristics are described by Wilkinson *et al.* (1954).

Growth medium. The organism was grown in a medium containing a relatively high sugar and low nitrogen-source concentration which gives maximal polysaccharide production (Wilkinson *et al.* 1954). Growth was carried out in 1 l. screw-capped round bottles, each containing 200 ml. of the following medium: 1·0% Na_2HPO_4; 0·3% KH_2PO_4; 0·03% $(NH_4)_2SO_4$; 0·1% K_2SO_4; 0·1% NaCl; 0·02% $MgSO_4$, $7H_2O$; 0·002% $CaCl_2$, $6H_2O$; 0·0001% $FeSO_4$; 1% carbohydrate (glucose, sucrose, galactose, mannitol, xylose, rhamnose, fucose or glucurone; in the case of glucurone, sufficient NaOH was added to adjust the pH to 7·3). The medium was sterilized by steaming and inoculated from a 24 hr. culture previously grown and adapted to the sugar to be used in the growth medium. The bottles were then filled with O_2 through sterile plugged tubing for 2 min. In order to maintain adequate oxygenation of the medium during growth, the bottles were rotated horizontally on a drum of horizontal axis capable of holding six bottles at a time and mounted in a 35° incubator. The cultures were grown for 48 hr.

General analytical methods

Total nitrogen was determined by the micro-Kjeldahl method. Total phosphorus was determined by the Fiske & Subbarow (1925) method. The ash content was determined by incinerating a little of the material in a Pt boat to constant weight, a drop of 60% (w/v) $HClO_4$ being added after the first ignition.

The reducing value of the unhydrolysed and hydrolysed polysaccharides was determined using Somogyi's (1945) copper reagent in conjunction with a colorimetric reagent (Nelson, 1944) calibrated against glucose. The results were expressed in terms of percentage glucose.

The anthrone value. The modified anthrone method of Fairbairn (1953) was adopted. The method, which is finding increasing application as a rapid and sensitive means of estimating polysaccharides, is of little direct value in composition studies. However, it was found to be useful as an independent check on the composition data derived by the methods described below. This was done by comparing the experimental anthrone value of a polysaccharide with the anthrone value calculated from the composition data. The 'anthrone equivalents' of the component sugars (colour given by 100 μg. of the sugar/colour given by 100 μg. glucose) were as follows: galactose, 0·60; fucose, 0·92; glucurone, 0·05. All results were expressed in terms of percentage glucose.

Determination of component sugars

Glucose, galactose and fucose. These sugars were estimated after hydrolysis and chromatographic separation on paper according to the general principles described by Flood, Hirst & Jones (1948). The polysaccharides were found to be polyuronides and resistant to the normal methods of hydrolysis such as N-H_2SO_4 at 100° for 24 hr. The conditions finally adopted as the standard method for quantitative studies which gave complete hydrolysis as indicated by a maximal reducing value, were 24 hr. in 96% (w/v) formic acid at 100°, followed by 6 hr. in N-H_2SO_4 at 100°. Samples of polysaccharide (20–40 mg.) were hydrolysed for 24 hr. with 96% formic acid (2 ml.) in a sealed tube in a boiling-water bath. (Preliminary experiments showed that a polysaccharide concentration within the range 10–20 mg./ml. gave minimal destruction.) A weighed quantity (15–30 mg.) of reference sugar (arabinose), shown chromatographically to be absent from the polysaccharide hydrolysate, was added and the formic acid distilled off under reduced pressure. Further hydrolysis was carried out for 6 hr. at 100° after the addition of N-H_2SO_4 (2 ml.). The contents of the tube were neutralized by the addition of $BaCO_3$. It was assumed that the destruction of the reference sugar and the component sugars of the polysaccharide took place at the same rate.

Because of the difficulties introduced by lactone trails on chromatograms and of the anomalous yield of formic acid from periodate oxidation, it was necessary to remove uronic acids from the hydrolysate. Preliminary experiments were made using anion-exchange resins, but these were discontinued because of the possibility that free sugars might be differentially adsorbed on to the resin (Roseman, Abeles & Dorfman, 1952). Finally it was found that the uronic acid could be removed from the neutral hydrolysate without disturbing the ratio of free sugars, by precipitation of the Ba salt. Ethanol (2 vol.) was added to the supernatant after $BaCO_3$ treatment, the tube was left to stand overnight at 4°, and the precipitated Ba salt of the uronic acid was removed by centrifuging. The supernatant was treated with Amberlite cation-exchange resin IR-120 and evaporated to dryness. The syrup was dissolved in an appropriate small volume of water and suitable amounts were applied to the starting line of a strip of Whatman no. 1 filter paper. The solvent used for separation in early experiments was a benzene–butanol–pyridine–water mixture (1:5:3:3, v/v) for 44 hr. at 15°. This gave good separation of sugars but often interfered in the estimations because of erratic high blank values. It was replaced in later experiments by butanol–ethanol–water mixture (5:1:4, v/v), which was found to give equally good separation after 120 hr. at 15° while having reproducible small blank values. After drying, the side strips were sprayed with saturated aqueous aniline oxalate, and the colours developed by heating; it was often found that spots invisible by daylight could be observed under ultraviolet radiation. The sugars were eluted from the corresponding central portion of the chromatogram by the method of Laidlaw & Reid (1950). The amounts of the sugars were estimated by periodate oxidation (Hirst & Jones, 1949).

Fucose. This was also determined separately on the unhydrolysed polysaccharide by the colorimetric method for methylpentoses (Dische & Shettles, 1948). The method was claimed by the authors to be specific for methylpentoses but in trial experiments it was found that all the sugar components of the polysaccharide would affect the result. The relative colour-producing powers of fucose, glucose and glucurone were 1·00, 0·058 and 0·021, respectively, and in each case the colour was proportional to the sugar concentration. Experiments with mixtures of these sugars showed that when the colours produced by glucose and glucurone were subtracted from the total colour, fucose (6–44 μg.) could be estimated in the presence of glucose (68 μg.) and glucurone (37 μg.) with recoveries of 95–112%. By correcting the results for the amounts of glucose and uronic acid (assumed to be glucurone) known to be present in the polysaccharide, the fucose content determined by this method was found to give results comparable to the chromatographic method.

Uronic acid. This was determined on the unhydrolysed polysaccharide by the decarboxylation method of McCready, Swenson & Maclay (1946).

Isolation and purification of the polysaccharide

The culture was centrifuged at 13000 rev./min. for 30 min. and the cellular centrifugate discarded. High-speed centrifuging is necessary because of the viscosity of the slime polysaccharide; the culture supernatant gave a relative viscosity of 22 at 15° as determined in an Ostwald viscometer. Acetone (1·5 vol.) was added to the supernatant and the mixture stirred with a spatula. The polysaccharide gel adhered to the spatula and could be washed and dehydrated by gradually increasing concentrations of acetone until the gel was converted into a stringy white solid. This resulted in a considerable purification of the polysaccharide, since many of the impurities present in the culture supernatant did not adhere to the spatula and were not precipitated by 1·5 vol. acetone (e.g. the extracellular 0-polysaccharide). The dehydrated polysaccharide was dissolved to give a 0·5% solution in an acetate buffer containing 4% (w/v) sodium acetate and 2% (v/v) acetic acid. It was then deproteinized by shaking each 100 ml. with six to eight successive lots of a mixture of $CHCl_3$ (20 ml.) and *n*-butanol (4 ml.) (Sevag, 1934). The final aqueous solutions were combined, recentrifuged and precipitated by acetone (1·5 vol.) as described previously. The dried precipitate was redissolved in water to give a 0·2% solution and was dialysed against running tap water for 96 hr. The purified polysaccharide was finally precipitated from solution by one of two methods.

Method A

The solution was shaken alternately with small portions of anion and cation-exchange resins (Amberlite IR-4B and

IR-120). This produced the free acid form of the polysaccharide which was no longer precipitable by acetone. Instead, it was lyophilized.

Method B

Sodium acetate (1%) was added followed by precipitation with acetone (1·5 vol.). The precipitate was washed with acetone and ether and dried in a vacuum desiccator over P_2O_5.

In the procedures described above, it was difficult to remove protein effectively from the polysaccharide preparation because of its high viscosity in solution. It was found that if a solution was boiled at a neutral pH, the viscosity fell to a low value with a consequent increase in the ease of handling. Consequently, the following method was adopted for later preparations.

Method C

The initial precipitate from the culture supernatant was dissolved in distilled water to give a 0·5% solution, the pH was adjusted to 7·0 and the solution was boiled for 30 min. The further stages of deproteinization and purification were as described in method *B*, except that the polysaccharide required the addition of 3 vol. of acetone followed by a period of 12 hr. at 4° to allow complete precipitation.

The yields of polysaccharide isolated by the three methods were of the order of 1 g./3·6 l. of culture.

Separation and identification of the component sugars of the polysaccharide

Polysaccharide (2 g.) was hydrolysed by heating on a boiling-water bath for 24 hr. in 96% formic acid (200 ml.). After removal of the formic acid by distillation under reduced pressure, the resultant syrup was heated at 100° for 6 hr. in N-H_2SO_4 (100 ml.). The product was neutralized with $BaCO_3$, concentrated to 30 ml. and methanol (60 ml.) was added. The mixture was left for 24 hr. at 4° and the precipitate of the Ba salt of the uronic acid was centrifuged. Chromatographic analysis showed that the supernatant contained glucose, fucose and a small amount of uronic acid. There was also a slight trace of an unknown sugar travelling on a chromatogram faster than fucose. This may have been fucoketose produced by prolonged contact with $BaCO_3$, since fucose treated with $BaCO_3$ for 24 hr. at room temperature produced a spot similar in its chromatographic behaviour. (Rate of movement relative to 2:3:4:6-tetramethyl glucose (R_G) in *n*-butanol–ethanol–water (5:1:4, v/v) was 0·36.) The supernatant was evaporated to a syrup and redissolved in water to 60 ml. The remaining uronic acid was removed by passage through a column of Amberlite IRA-400 resin previously converted into the carbonate form. The eluate (500 ml.) containing glucose and fucose, was concentrated and dried over P_2O_5 to give a syrup (1·5 g.), which was fractionated on a cellulose column at 37° by using as the solvent butanol saturated with water as described by Hough, Jones & Wadman (1949). Every tenth tube was examined for the presence of carbohydrate by the anthrone method and for the nature of the sugar chromatographically. The following results were obtained: tubes 1–54, no carbohydrate; tubes 55–95, fucose; tubes 96–124, fucose and glucose; tubes 125–260, glucose.

Identification of L-*fucose.* The syrup (93 mg.) from tubes 55–95 had $[\alpha]_D^{15} - 69°$ in water (*c*, 2) (literature value $-76°$) and paper chromatography showed the presence of fucose only. The identity of the sugar as L-fucose was confirmed by the formation of L-fucose phenylhydrazone (m.p. and mixed m.p. 156–159°).

Identification of D-*glucose.* Syrup (300 mg.) from tubes 125–245 had $[\alpha]_D^{15} + 50°$ in water (*c*, 2) (literature value, $+52·5°$) and paper chromatography showed the presence of glucose only. The identity of the sugar as D-glucose was confirmed by the formation of β-D-glucose penta-acetate (m.p. and mixed m.p. 124–127°).

Identification of the uronic acid. This will be dealt with in a later communication. The fact that a lactone was formed, indicated that the uronic acid was not galacturonic acid.

Analysis of the extracellular polysaccharide from glucose-grown Aerobacter aerogenes

The basic results of the quantitative analysis of the polysaccharide prepared by methods *A*, *B* and *C* after growth on glucose are shown in Table 1. The main components are glucose, fucose and uronic acid. A small amount of galactose was also present but was most likely due to contamination by some extracellular O-polysaccharide which is probably a galactan (Dudman & Wilkinson, unpublished results). The colorimetric method for the estimation of fucose was found to give more consistent results than the chromatographic method, possibly because of the rather diffuse nature of the fucose spot on the chromatograms. The anthrone value calculated from the analytical data compares well with the experimental result, showing that all the anthrone-reactive carbohydrates in the polysaccharide had been accounted for. It can be seen that the relative proportions of the main component sugars remain constant irrespective of the mode of preparation, showing that boiling the polysaccharide solution caused no change in composition in spite of the great lowering in viscosity.

Further analysis was carried out on the polysaccharide prepared by method *A*. The specific rotation was $[\alpha]_D^{15} - 50° \pm 5°$ in water (*c*, 0·1). The uronic acid content, determined by the naphthoresorcinol method (Jarrige, 1947), was 22%. It was found over a range of different heteropolysaccharides, that this method gave consistently low values compared with those by decarboxylation, possibly owing to difficulty in obtaining complete hydrolysis of the polysaccharide without decomposition of the uronic acid. The acid equivalent was 760, corresponding to a uronic acid content of 25·5%. The reducing value after hydrolysis was 78%. Amino acids and hexosamines were absent as indicated by negative results after paper chromatography using respectively, ninhydrin and the Elson & Morgan (1933) reagent as developers. Ketoses were absent, as indicated by negative results after paper chromatography using naphthoresorcinol reagent as developer and also by the Seliwanoff reaction on the intact polysaccharide.

Analysis of the extracellular polysaccharide of Aero. aerogenes *after growth on various carbon sources*

The results of analysis of the extracellular polysaccharide produced by *Aero. aerogenes* after growth on a variety of carbohydrates as the sole carbon and energy source and isolated by method *C*, are shown in Table 1. The carbohydrates were chosen to give representative types of sugars as well as those normally occurring in the polysaccharide. The proportions of the component sugars, glucose, fucose and uronic acid, can be seen to be identical within the limits of experimental error in each of the polysaccharides.

It has been reported that *Bacillus polymyxa* when grown on monosaccharides produces an extracellular polyuronide while after growth on sucrose it produces a polyuronide together with an extracellular levan (Hestrin, Avineri-Shapiro & Aschner, 1943; Forsyth & Webley, 1949). Consequently, *Aero. aerogenes* was grown on sucrose as the sole source of carbon and energy, and the polysaccharide isolated according to method *C*. A full analysis was not made but fructose was shown to be absent by paper chromatography after mild acid hydrolysis and by the Seliwanoff reaction on the intact polysaccharide.

DISCUSSION

All extracellular heteropolysaccharides produced by bacteria from widely different species and examined with the aid of modern techniques, have been found to contain uronic acid or hexosamine components. The extracellular polysaccharide of *Aero. aerogenes* A 3 (S 1) is a polyuronide and therefore no exception to this rule. An unusual feature is the presence of L-fucose. Until a recent report by Norris, de Sipin, Zilliken, Harvey & György (1954) that fucose was a component of the polysaccharide of a mucoid mutant of *Lactobacillus bifidus*, fucose has never been found in a bacterial polysaccharide. However, another naturally occurring methylpentose, rhamnose, has been identified in the polysaccharides of a variety of strains (see Whistler & Smart, 1953).

An ultracentrifugal examination of the polysaccharide, prepared by method *A* after growth on glucose, indicated the presence of only one component with a narrow molecular weight distribution, thus suggesting the presence of a single polysaccharide rather than a mixture of two or more molecular species (C. T. Greenwood, 1954; unpublished results).

The amount of the three main components, glucose, fucose and uronic acid, was found to be independent of the carbon source used in the growth medium. This has a bearing on the mode of

Table 1. *Analytical results on the polysaccharide of* Aerobacter aerogenes *after growth on various carbon sources*

	Sugar in growth medium									
	Glucose			Galactose	Mannitol	Xylose	Rhamnose	Fucose	Glucarone
Method of preparation	*A*	*B*	*C*	*C*	*C*	*C*	*C*	*C*	*C*
Glucose (%)	46	46	50	45	43	49	47	44	46
Galactose (%)	2	2	1	1	2	2	2	2	1
Fucose (%)									
(*a*) Chromatographically	10		10	7	4	8	8	7	7
(*b*) Colorimetrically	9		10	9	9	10	9	9	9
Uronic acid (%)	27	28	29	28	25	28	26	27	29
Total % sugar components	84	85	90	83	79	89	84	82	85
Fucose } relative to	20	20	20	20	21	20	19	20	20
Uronic acid } glucose as 100	58	61	58	61	58	58	56	61	62
Reducing value of unhydrolysed polysaccharide	1·8	1·6	0·7	0·8	1·4	1·3	0·8	1·2	1·3
Anthrone value (%)	54	55	64	59	53	60	52	55	57
Anthrone value theoretical (%)	56	56	60	54	53	60	57	55	56
Ash (%)	1·3	4·0	7·6	7·0	6·3	4·5	4·0	3·7	2·9
N (%)	1·05	0·18	0·17	0·15	0·15	0·39	0·79	0·65	0·89
P (%)	0·10	0·12	0·04	0·04	0·06	0·19	0·49	0·45	0·43

synthesis of such heteropolysaccharides. Work on homopolysaccharides summarized by Barker & Bourne (1953) and by Hassid (1954) has shown that they are synthesized according to the general equation:

$$Gt\text{–}O\text{–}X + H\text{–}O\text{–}Gr \rightleftharpoons Gt\text{–}O\text{–}Gr + X\text{–}O\text{–}H,$$

where Gt–O and X are, respectively, the sugar residue and the aglycone portion of a glycoside donor (Gt–O–X) which serves as the substrate for an enzyme, and Gr–O–H is the carbohydrate receptor molecule. Two main types of compounds have been demonstrated as glycoside donors, (1) disaccharides and higher saccharides where X is a carbohydrate residue, (2) sugar phosphates where X is a phosphate residue. However, no evidence has been obtained as to the nature of the glycoside donors in heteropolysaccharide formation although they may be assumed to function in a similar manner. By varying the nature of the carbon source during growth and polysaccharide formation, one can be assumed to vary the equilibrium concentrations of these glycoside donors so that the relative proportions are different. This follows unless all the carbon sources are metabolized to a common intermediate from which all the glycoside donors are formed. If polysaccharide synthesis is carried out by a comparatively non-selective enzyme or enzymes which string together glycoside radicals according to the availability of glycoside donors, then the nature of the polysaccharide should vary according to the concentration of glycoside donors and thus according to the nature of the carbon growth source. This was not so in the case of the extracellular heteropolysaccharide of *Aero. aerogenes* and therefore a mechanism of this type is unlikely to occur.

Rather, there appear to be two possible mechanisms:

(1) Synthesis carried out by a series of completely specific enzymes. The number of enzymes involved would depend on the complexity of the polysaccharide; even for the simplest type of regular structure (e.g. a linear chain with regularly spaced branching points) at least three specific enzymes would be required. Preliminary results of a structural investigation of the extracellular polysaccharide of *Aero. aerogenes* indicate a highly branched complex molecule, and therefore that a large number of enzymes would be required in order to synthesize this polysaccharide invariably.

(2) Synthesis carried out by a template mechanism as envisaged for protein synthesis. Such a template would probably involve deoxyribonucleic acid in view of the evidence of type transformation in the pneumococcus and other organisms (summarized by Austrian, 1952).

Since the ability to synthesize an extracellular polysaccharide and therefore form a mucoid colony is apparently controlled by a single mutable step, and thus according to current views by one enzyme, the former mode of synthesis involving a large number of enzymes is less likely. Thus the evidence favours the idea that heteropolysaccharide synthesis is carried out by some template mechanism, and is thus different from the mechanism of homopolysaccharide formation.

SUMMARY

1. The extracellular slime polysaccharide of *Aerobacter aerogenes* A 3 (S 1) (Klebsiella Type 54) was isolated and purified and shown to contain three main component sugars: D-glucose (50%), L-fucose (10%) and an unidentified uronic acid (29%).

2. The bacterium was grown in presence of a variety of carbohydrates as the sole carbon and energy source and the polysaccharides produced from each were isolated. The composition was found to be invariable. The implications of this fact upon the nature of heteropolysaccharide synthesis is discussed.

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The Preparation and Properties of β-Glucuronidase

7. ACTION ON ESTER GLUCURONIDES

By G. A. LEVVY and J. T. WORGAN
Rowett Research Institute, Bucksburn, Aberdeenshire

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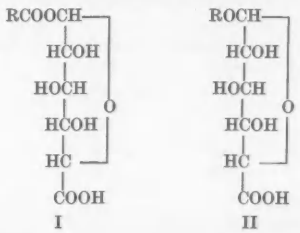
It has long been known that aromatic carboxylic acids can be conjugated with glucuronic acid in the animal body to form ester glucuronides (general formula, I; for review, see Williams, 1949): the best-known example is benzoyl glucuronide (I, R = C_6H_5), but the glucuronide of veratric acid (3:4-dimethoxybenzoic acid) is more easily prepared (Sammons & Williams, 1946). Interest in the ester glucuronides has recently been stimulated by the discovery that the branched-chain fatty acids, α-ethylbutyric acid and α-ethylhexanoic acid, can form derivatives of this type *in vivo* (Kamil, Smith & Williams, 1953). Unlike the ether glucuronides formed by alcohols and phenols (general formula, II), the ester glucuronides in general are unstable in alkaline solution and therefore give a positive reaction in tests for reducing sugars.

The biosynthetic glucuronides of alcohols and phenols are hydrolysed by the enzyme β-glucuronidase, but the action of this enzyme on ester glucuronides has not hitherto been studied, unless the synthetic compound β-glucuronic acid 1-phosphate (II, R = H_2PO_3; Levvy & Marsh, 1952), is regarded as a member of the group. At the suggestion of Professor R. T. Williams, specimens of α-ethylbutyryl, α-ethylhexanoyl and veratroyl glucuronides which he supplied were examined as possible substrates for mouse-liver β-glucuronidase.



I II

EXPERIMENTAL AND RESULTS

Methods

In general, methods followed those used in earlier papers, in particular, Levvy & Marsh (1952) and Levvy (1954).

Inhibition of the hydrolysis of phenolphthalein glucuronide

α-Ethylbutyryl, α-ethylhexanoyl and veratroyl glucuronides were tested as competing substrates in the hydrolysis of phenolphthalein glucuronide by mouse-liver β-glucuronidase (Levvy & Marsh, 1952). As a test of enzyme specificity, this technique is less dependent on the purity of the enzyme preparation than direct measurements of hydrolysis. In the present instance, the difficulty to be avoided was the possible presence in the liver preparations of an esterase capable of hydrolysing ester glucuronides. To conserve supplies of the ester glucuronides, the microprocedure for enzyme assay described in connexion with baicalinase (Levvy, 1954) was adopted. Incubation was for 1 hr. at 37° and pH 5·2 in sodium acetate–acetic acid buffer, final concentration 0·125N. The phenolphthalein glucuronide concentration ranged from 0·00125 to 0·000031M.

All three ester glucuronides depressed the release of phenolphthalein and acted competitively, the percentage depression varying inversely with the phenolphthalein glucuronide concentration. Values for K_i, the dissociation constant for the complex formed between enzyme and competing compound, were determined by the method of Lineweaver & Burk (1934). The mean values obtained were: α-ethylbutyryl glucuronide, $7{\cdot}9 \times 10^{-4}$M; α-ethylhexanoyl glucuronide, $2{\cdot}2 \times 10^{-4}$M; veratroyl glucuronide, $3{\cdot}5 \times 10^{-5}$M. The reciprocals of these figures give an approximate measure of the affinities of the respective ester glucuronides for the enzyme (Levvy & Marsh, 1954). For phenolphthalein glucuronide and mouse-liver β-glucuronidase, the

dissociation constant is $1{\cdot}3 \times 10^{-4}$M (Levvy, 1952). One should mention the corresponding figure for glucuronic acid, 16×10^{-4}M, since this product of glucuronide hydrolysis inhibits the enzyme (Spencer & Williams, 1951): the affinity, however, is too low for its presence to account for the effects of the ester glucuronides.

Hydrolysis of α-ethylhexanoyl glucuronide

It was found possible to extract α-ethylhexanoyl glucuronide from acidified aqueous solutions with ethyl acetate, and thus to follow its enzymic hydrolysis by measuring the glucuronic acid liberated, since this is not extracted. The procedure was similar to that used for following the hydrolysis of flavone glucuronides by mouse-liver β-glucuronidase (Levvy, 1954), terminating in the cerimetric estimation of reducing sugar (Levvy, 1946). α-Ethylhexanoic acid did not interfere in this estimation, but in any case it was extracted by ethyl acetate. The ester glucuronide in pure solution, incidentally, had 60% of the reducing power of an equivalent weight of free glucuronic acid for alkaline ferricyanide.

From the results shown in Table 1, it can be seen that the ester glucuronide was rapidly hydrolysed by a mouse-liver β-glucuronidase preparation, and that hydrolysis was inhibited by boiled saccharate solution (saccharo-1:4-lactone; see Levvy, 1952). In a concentration of $2{\cdot}5 \times 10^{-4}$M, saccharate caused 88% inhibition with $2{\cdot}5 \times 10^{-3}$M substrate. In another experiment, in which the saccharate concentration was doubled and the substrate concentration halved, there was no demonstrable liberation of reducing sugar in presence of the inhibitor. For purposes of comparison, Table 1 shows a parallel enzyme hydrolysis of menthol β-glucuronide. The lower affinity of this compound for the enzyme (dissociation constant 4×10^{-3}M; Fishman, 1939) as compared with α-ethylhexanoyl glucuronide is reflected in the more powerful effect of saccharate.

pH-Optimum for α-ethylhexanoyl glucuronide

The measurement of reducing material not extracted by ethyl acetate was applied to determinations of the pH-optimum for hydrolysis of α-ethylhexanoyl glucuronide by a mouse-liver β-glucuronidase preparation. At each point, the enzyme activity was determined in duplicate, and the usual controls were done for the effects of enzyme and substrate on the pH, and for the non-extractable reducing material present after incubation of each alone. Owing to the errors inherent in the method, individual results were not entirely satisfactory. Different experiments were, however, consistent in showing the presence of two peaks in the pH/activity curve, at pH 4·4 and pH 5·1–5·2 (Fig. 1). The curve as a whole thus resembles very closely those obtained for the hydrolysis of glucuronides of alcohols and phenols by β-glucuronidase, with optima at pH 4·5 and pH 5·0–5·2 (Mills, 1948; Kerr, Graham & Levvy, 1948).

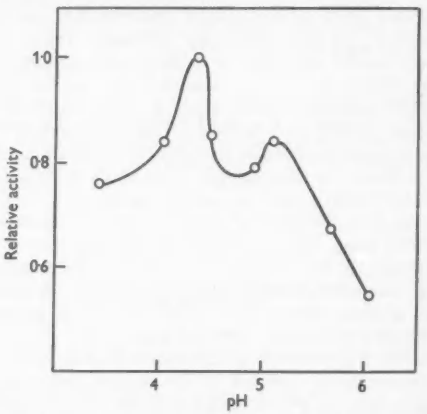


Fig. 1. Effect of pH on the hydrolysis of 0·0025M α-ethylhexanoyl glucuronide in 0·125N sodium acetate–acetic acid buffer after 2 hr. incubation at 37°.

Table 1. *Liberation of reducing sugar from α-ethylhexanoyl glucuronide by mouse-liver β-glucuronidase*

Hydrolysis mixture incubated for 2 hr. at 37° and pH 5·2 in sodium acetate–acetic acid buffer, final concentration 0·125N. Saccharate solution boiled for 30 min. before use.

Substrate	Inhibitor	Glucuronic acid liberated (μg.)*	Hydrolysis (%)	Inhibition (%)
0·0025M α-Ethylhexanoyl glucuronide	None	453	58	—
0·0025M α-Ethylhexanoyl glucuronide	0·00025M Saccharate	55	7	88
0·0025M α-Ethylhexanoyl glucuronide	0·00001M Saccharate	308	40	32
0·01M Menthol β-glucuronide	None	169†	5	—
0·01M Menthol β-glucuronide	0·00001M Saccharate	70†	2	59

* Total in incubation mixture (1·6 ml.).
† Enzyme preparation diluted to one-third.

β-Glucuronidase as an esterase

If β-glucuronidase hydrolysed ester glucuronides, the question arose as to whether it had any general properties as an esterase. A number of common esters were therefore examined as competing substrates for mouse-liver β-glucuronidase in the hydrolysis of 0·00063M phenolphthalein glucuronide in 0·125N-sodium acetate–acetic acid buffer, pH 5·2 (Levvy & Marsh, 1952). All the test compounds were in a final concentration of 1 mg./ml. None of them had any effect, i.e. if it is an esterase, β-glucuronidase is specific for compounds in which the alcohol radicle is glucuronic acid. The compounds tested were: ethyl acetate, methyl oleate, sodium β-glycerophosphate, glyceryl monoacetate, glyceryl diacetate, tributyrin, tricaproin, glyceryl monostearate and glyceryl monoricinoleate.

DISCUSSION

The ester glucuronides of α-ethylbutyric, α-ethylhexanoic and veratric acids had high affinities for mouse-liver β-glucuronidase: in the case of veratroyl glucuronide the affinity was higher than that of phenolphthalein glucuronide. α-Ethylhexanoyl glucuronide was rapidly hydrolysed by partially purified β-glucuronidase preparations, and the peaks on the pH/activity curve coincided with those for ether glucuronides. Saccharo-1:4-lactone caused almost complete inhibition of the hydrolysis of α-ethylhexanoyl glucuronide. From these experiments it may be concluded that β-glucuronidase hydrolyses ester glucuronides. Since the enzyme is specific for β-glucopyranuronides (Levvy & Marsh, 1952), this conclusion supports the generally accepted structure (I) for the ester glucuronides. β-Glucuronidase, however, did not appear to possess general esterase properties.

Evidence for the unity of a group-specific enzyme can never be anything else but circumstantial, subject always to the possibility that the enzyme may be physically separated into components with different specificities. While the ester glucuronides behaved in every respect like any other substrate for β-glucuronidase, one must admit that the experimental findings are open to an alternative explanation. Hydrolysis of the ester glucuronides could have been partially or entirely due to a second enzyme in the β-glucuronidase preparations if the following three assumptions are made: (i) that the pH/activity curve resembles that for β-glucuronidase, (ii) that the second enzyme is powerfully inhibited by saccharo-1:4-lactone, (iii) that ester glucuronides are competitive inhibitors in the general sense for β-glucuronidase. On such assumptions as these, however, a distinct enzyme could be postulated for the hydrolysis of every individual glucuronide.

SUMMARY

1. The ester glucuronides of α-ethylbutyric, α-ethylhexanoic and veratric acids had high affinities for mouse-liver β-glucuronidase, as measured by their effects in depressing the hydrolysis of phenolphthalein glucuronide by the enzyme.

2. α-Ethylhexanoyl glucuronide was rapidly hydrolysed by β-glucuronidase preparations. The pH/activity curve was similar to those for ether glucuronides, and hydrolysis was inhibited by saccharo-1:4-lactone.

3. β-Glucuronidase did not appear to possess general esterase properties.

4. On the basis of these findings it was concluded that ester glucuronides are substrates for β-glucuronidase.

Mr A. McAllan is thanked for technical assistance.

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Animal Fats

5. THE COMPONENT ACIDS OF CHIMPANZEE FAT*

By F. D. GUNSTONE
Chemistry Department, University of Glasgow†

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Earlier papers in this series have dealt with the component acids of fats from several animals, and the present paper is concerned with chimpanzee fat, which does not appear to have been examined previously.

EXPERIMENTAL

The crude material obtained (540 g.) was autoclaved at 120° and extracted as previously described (Gunstone & Russell, 1954*a*) yielding a solid fat (465 g.) of iodine value 58·1, saponification equivalent 280·1, and free acid 18·9% (as oleic). The mixed acids obtained on hydrolysis had iodine value 60·5 and equivalent 270·7.

These acids were separated into three fractions by crystallization from methanol at −55 to −60° and subsequent recrystallization of the insoluble portion at −20°. Each fraction was then esterified and distilled and the ester fractions examined as described in the earlier papers of this series. The results are given in Tables 1 and 2.

Myristic acid (m.p. 51–53°), palmitic acid (m.p. 61–63°), and stearic acid (m.p. 69–71°) were isolated from appropriate fractions and the presence of hexadec-9-enoic, oleic, and linoleic acid confirmed by the preparation of 9:10-dihydroxypalmitic (m.p. 124–126°), 9:10-dihydroxystearic (m.p. 128–131°) and 9:10:12:13-tetrabromostearic acid (m.p. 112–113°) respectively. No hexabromostearic acid was obtained, confirming the absence of linolenic acid. (All these samples gave no depression of m.p. when mixed with an authentic specimen.)

DISCUSSION

In Table 3 the results obtained in the present work are compared with those obtained for other herbivorous animals for the C_{16} and C_{18} acids only, the fats being listed in order of their iodine value. These figures clearly show that as the iodine value rises

* Paper 4 of this series: Gunstone, F. D. & Russell, W. C. (1954*b*).

† Present Address: Chemistry Department, University of St Andrews.

Table 1. *Low-temperature crystallization of chimpanzee fatty acids*

Fraction	Acids: Wt. (g.)	Acids: % (w/w)	Acids: Iodine value	Esters: Iodine value	Esters: Sapon. equiv.
A Insoluble at −20°	64·6	35·8	3·0	2·5	275·9
B Soluble at −20°, insoluble at −55°	74·9	41·5	83·0	79·3	292·2
C Soluble at −55°	40·9	22·7	111·1	104·0	—

Table 2. *Component acids of chimpanzee fat*

All values except in last column are % (w/w) of total.

	Fraction *A*	Fraction *B*	Fraction *C*	Total	% (wt.)‡	% (mol.)‡
Lauric	—	—	0·37	0·37	0·4	0·5
Myristic	0·66	1·05	0·68	2·39	2·4	2·8
Palmitic	27·10	2·42	0·21	29·73	29·8	31·4
Stearic	6·75	—	—	6·75	6·8	6·4
Arachidic	0·17	—	—	0·17	0·2	0·2
Dodecenoic	—	—	0·32	0·32	0·3	0·4
Tetradecenoic	—	0·10	0·72	0·82	0·8	1·0
Hexadecenoic	0·05	1·59	3·33	4·97	5·0	5·3
Hexadecadienoic	—	—	0·15	0·15	0·1	0·2
Octadecenoic	1·02	35·77	7·60	44·39	44·5	42·6
Octadecadienoic	—	0·50	7·44	7·94	8·0	7·7
As eicosenoic§	—	—	1·67	1·67	1·7	1·5
Unsaponifiable	0·05	0·07	0·21	0·33	—	—

‡ Excluding unsaponifiable material.
§ Includes all unsaturated acids higher than C_{18} (average unsaturation −2·0 H).

Table 3. *Component acids of some herbivorous animal fats*

All values are % (w/w).

	Iodine value	Saturated		Unsaturated				Total saturated	Total C_{18} acids
		C_{16}	C_{18}	C_{16} (−2·0 H)	C_{18} (−2·0 H)	C_{18} (−4·0 H)	C_{18} (−6·0 H)		
Camel[1]	35·1	29	27	3	26	2	1	64	56
Deer[1]	35·5	25	35	3	25	3	3	66	66
Ox[2]	43·2	29	21	3	41	2	—	54	64
Sheep[3]	43·4	25	28	1	37	6	—	56	71
Sheep[3]	49·1	28	16	1	47	4	—	47	67
Kangaroo[4]	50·1	26	14	3	45	3	—	47	62
Pig[5]	54·3	30	16	3	41	7	—	47	64
Chimpanzee	58·1	30	7	5	45	8	—	40	60
Pig[5]	60·0	28	12	3	48	6	—	41	66
Giant Panda[4]	64·8	26	7	4	45	12	—	38	64

[1] Gunstone & Paton (1953); [2] Hilditch & Longenecker (1937); [3] Hilditch & Pedelty (1941); [4] Hilditch, Sime & Maddison (1942); [5] Hilditch, Lea & Pedelty (1939).

(35–65) the total content of saturated acids falls steadily (65–40 %). This is entirely due to the decreasing amount of stearic acid (35–7 %), the content of palmitic acid being constant (25–30 %) throughout the range (cf. Banks & Hilditch, 1931). The total amount of C_{18} acids also remains fairly constant (60–70 %) for as the iodine value rises the decrease in stearic acid is balanced by an increase in the amount of oleic acid and, in the higher range of iodine value (55–65), by the appearance of larger amounts of octadecadienoic acid.

The values obtained for this sample of chimpanzee fat fit well into this general scheme.

SUMMARY

Chimpanzee fat contains palmitic (30 %) and oleic acid (45 %) as major components with octadecadienoic (8 %), stearic (7 %), and hexadecenoic acid (5 %) as the most important minor component acids. It is similar to fats of similar iodine value from other herbivorous animals.

The author is grateful to Mr E. C. Appleby, of the Zoological Park, Edinburgh, who supplied the fat used in this investigation.

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Animal Fats

6. THE COMPONENT ACIDS OF TIGER FAT AND OF PUMA FAT

By F. D. GUNSTONE
*Chemistry Department, University of Glasgow**

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This communication is concerned with the component acids of tiger fat (*Felix tigeris*) and of puma fat (*F. concolor*). Despite the fact that the composition of fats from these animals has already been reported (for references, see Table 5) it was considered of interest to examine the samples available in view of their higher iodine value.

* Present address: Chemistry Department, University of St Andrews.

EXPERIMENTAL

These two fat samples were obtained through the kindness of Professor T. F. Hewer from animals which had died in Bristol Zoological Gardens. One sample was taken from the subcutaneous region of the groin of a 14-month-old male tiger, whilst the other came from the abdominal wall, the mesentery, and the omentum of an 11-year-old female puma which had been reared in captivity in this country. Both animals were fed on a mixture of raw cow and horse

flesh; the wild members of the species live mainly on deer and sheep.

The fats were treated by the methods described in earlier papers in this series and the mixed acids were separated into three fractions by low-temperature crystallization, each ester fraction being examined as before. The results are set out in Tables 1–4.

Identification of acids

Samples of myristic (m.p. 52–54°), palmitic (m.p. 60–63°), and stearic acid (m.p. 68–71°) were isolated from appropriate fractions of the tiger fat analysis and the presence of hexadec-9-enoic, oleic, and linoleic acid confirmed by the preparation of 9:10-dihydroxypalmitic (m.p. 124–125°), 9:10-dihydroxystearic (m.p. 129–131°), and 9:10:12:13:15:16-hexabromostearic acid (m.p. 181–184°). None of these melting points were depressed when samples were mixed with specimens of the authentic compound. Attempts to isolate the 9:10:12:13-tetrabromostearic acid characteristic of linoleic acid were unsuccessful; from a series of crystallizations several fractions melting within the range 85–100° were obtained the analysis of which showed them to be essentially tetrabromostearic acid but it is doubtful whether any fraction was entirely homogeneous.

Myristic (m.p. 51–54°), palmitic (m.p. 61–63°), stearic (68–71°), 9:10-dihydroxypalmitic (m.p. 123–125°), 9:10-dihydroxystearic acid (m.p. 129–131°) and 9:10:12:13:15:16-

Table 1. *Characteristics of tiger fat and of puma fat*

	Fat			Mixed acids	
Animal	Iodine value	Sapon. equiv.	Free acid (as % oleic)	Iodine value	Sapon. equiv.
Tiger[1]	56·8	286·4	—	—	—
Tiger[2]	49·2	286·5	7·9	50·6	—
Tiger[3]	70·5	284·6	1·6	73·9	271·7
Puma[4]	38·8	324·7	1·8	46·7	278·7
Puma[3]	69·7	283·6	1·9	72·7	272·9

[1] Pathak & Godbole (1945); [2] Pathak & Agarwal (1952); [3] Present work; [4] Giral (1945).

Table 2. *Low-temperature crystallization of the mixed fatty acids*

		Acids			Esters	
	Fraction	Wt. (g.)	% (w/w)	Iodine value	Iodine value	Sapon. equiv.
		Tiger				
A	Insoluble at −20°	26·51	35·0	1·7	1·6	—
B	Soluble at −20°, insoluble at −45°	22·85	30·2	82·4	78·6	—
C	Soluble at −45°	26·29	34·8	137·9	131·6	
		Puma				
A	Insoluble at −20°	46·0	34·4	1·5	1·6	—
B	Soluble at −20°, insoluble at −45°	43·7	32·6	84·7	80·2	290·7
C	Soluble at −45°	44·2	33·0	133·1	125·6	290·2

Table 3. *Component acids of tiger fat*

All values except in last column are % (w/w) of total.

	Fraction				%	%
	A	*B*	*C*	Total	(wt.)*	(mol.)*
Myristic	0·69	0·57	1·75	3·01	3·0	3·6
Palmitic	22·89	2·57	1·29	26·75	26·8	28·4
Stearic	10·64	—	—	10·64	10·7	10·2
Tetradecenoic	—	0·16	0·42	0·58	0·6	0·7
Hexadecenoic	0·04	1·22	4·34	5·60	5·6	6·0
Hexadecadienoic	—	—	0·21	0·21	0·2	0·2
Hexadecatrienoic	—	—	0·16	0·16	0·2	0·2
Octadecenoic	0·61	24·89	12·43	37·93	38·1	36·6
Octadecadienoic	—	0·72	5·47	6·19	6·2	6·0
Octadecatrienoic	—	—	6·78	6·78	6·8	6·6
As eicosenoic†	—	—	1·74	1·74	1·8	1·5
Unsaponifiable	0·13	0·07	0·21	0·41	—	—

* Excluding unsaponifiable material.

† Includes all unsaturated acids higher than C_{18} (average unsaturation −4·7 H).

hexabromostearic acid (m.p. 181–182°), none of which depressed the melting point of an authentic specimen, were similarly isolated or prepared from appropriate fractions of the puma fat analysis. A crude sample of 9:10:12:13-tetrabromostearic acid (softens at 86°, melts at 106–109°; when mixed with an authentic specimen softens at 85°, melts at 109–112°) was also prepared.

DISCUSSION

In general terms the two fats reported in this paper are very similar in composition. Both contain oleic (38–40 %), palmitic (24–27 %), and stearic acid (10–11 %) as major components. The minor components include octadecadienoic acid (6–9 %), octadecatrienoic (4–7 %), hexadecenoic (5–6 %), myristic (3–4 %) and acids higher than C_{18} (2–3 %). The main difference between these two lies in the relative amounts of octadecadienoic and octadecatrienoic acid (see Table 5).

These results differ considerably from those previously recorded for the fats of these and other carnivorous animals (Table 5). The content of palmitic acid is fairly constant at about the usual level apart from one analysis of tiger fat but myristic acid is slightly higher than in the previous analyses of tiger and of puma fat. The amount of stearic acid (10–11 %) is considerably less than reported hitherto with the result that the total of the saturated acids (about 40 %) is lower than in the other analyses (46–54 %). Hexadecenoic acid falls between the lower values quoted for lion and cat (2–4 %) and the higher values of the earlier analyses of tiger and puma fat (7–13 %). The most interesting feature of these results is the presence of appreciable quantities of polyethenoid C_{18} acids (12–13 %) which seldom exceed 10 % in land animals; the quantity of octadecatrienoic is particularly interesting and recalls the much higher values for wild rabbit (42 %, Shorland, 1953) and horse (16 %, Gupta & Hilditch, 1951; 13–20 %, Shorland, Bruce & Jessop, 1952). The triene acid is essentially linolenic acid but the composition of the diene acid is less certain; puma fat probably contains some linoleic acid along with isomeric diene acids but no evidence of linoleic acid among the C_{18} diene acids of tiger fat was obtained.

Table 4. *Component acids of puma fat*

All values except in last column are % (w/w) of total.

	Fraction					
	A	*B*	*C*	Total	% (wt.)*	% (mol.)*
Myristic	0·98	1·44	1·70	4·12	4·1	4·9
Palmitic	21·54	1·89	0·67	24·10	24·2	25·7
Stearic	10·47	—	—	10·47	10·5	10·1
Arachidic	0·78	—	—	0·78	0·8	0·7
Tetradecenoic	0·01	0·05	0·79	0·85	0·9	1·0
Hexadecenoic	0·02	1·08	3·45	4·55	4·6	4·9
Octadecenoic	0·48	26·54	12·27	39·29	39·5	38·1
Octadecadienoic	—	1·08	7·42	8·50	8·6	8·3
Octadecatrienoic	—	—	3·59	3·59	3·6	3·5
As eicosenoic†	—	0·42	2·76	3·18	3·2	2·8
Unsaponifiable	0·12	0·10	0·35	0·57	—	—

* Excluding unsaponifiable material.
† Includes all unsaturated acids higher than C_{18} (average unsaturation −3·4 H).

Table 5. *Component acids of some carnivorous animal fats*

All values are % (w/w).

	Saturated				Unsaturated					
	C_{14}	C_{16}	C_{18}	C_{20}	C_{14} (−2·0 H)	C_{16} (−2·0 H)	C_{18} (−2·0 H)	C_{18} (−4·0 H)	C_{18} (−6·0 H)	C_{20}*
Lion[1] (*Panthero leo*)	4·9	28·9	17·8	0·1	0·6	1·9	40·3	—	—	3·0
Cat[2] (*Felix catus*)	3·6	29·2	16·6	—	1·2	4·3	40·8	1·9	—	Trace
Tiger[3] (*F. tigeris*)	1·3	12·8	26·8	5·0	—	—	54·1	—	—	—
Tiger[4]	1·0	22·5	24·6	1·2	0·6	7·1	38·9	4·1	—	—
Tiger[5]	3·0	26·8	10·7	—	0·6	5·6	38·1	6·2	6·8	1·8
Puma[6] (*F. concolor*)	1·3	22·4	26·9	3·7	—	12·6	26·2	2·3	—	4·6
Puma[7]	4·1	24·2	10·5	0·8	0·9	4·6	39·5	8·6	3·6	3·2

* Includes all unsaturated acids higher than C_{18}.
[1] Hilditch, Sime & Maddison (1942), also decanoic, 1·4 %; lauric, 1·1 %; [2] Hilditch *et al.* (1942), also lauric, 2·4 %; [3] Pathak & Godbole (1945); [4] Pathak & Agarwal (1952); [5] present work, also hexadecadienoic, 0·2 %; hexadecatrienoic, 0·2 %; [6] Giral (1945); [7] present work.

Table 6. *Relation between diet and fatty acid composition*

All values % (w/w) except for iodine values.

		Iodine value	Saturated		Unsaturated			
			C_{16}	C_{18}	C_{16} (−2·0 H)	C_{18} (−2·0 H)	C_{18} (−4·0 H)	C_{18} (−6·0 H)
Dietary animals	Deer[1]	35·5	25	35	3	25	3	2
	Sheep[2]	41·2	25	31	—	36	4	—
Carnivores fed on above	Puma[3]	38·8	22	27	13	26	2	—
	Tiger[4]	49·2	23	25	7	39	4	—
Dietary animal	Horse[5]	95·6	26	5	7	34	5	16
Carnivores fed on above	Puma[6]	69·7	24	11	5	40	9	4
	Tiger[6]	70·5	27	11	6	38	6	7

[1] Gunstone & Paton (1953); [2] Collin, Hilditch & Lea (1929); [3] Giral (1945); [4] Pathak & Agarwal (1952); [5] Gupta & Hilditch (1951); [6] present work.

In an earlier paper (Gunstone, 1955) it was stated that the fall in stearic acid content which accompanies the increase in iodine value over a range of land animal fats is balanced by an increase in oleic acid and, in the higher range of iodine value, by the appearance of large amounts of octadecadienoic acid. This is borne out by the present results if total polyethenoid C_{18} acids be substituted for octadecadienoic acid.

It is now recognized that animal fat is a mixture of synthesized and assimilated fat, the relative amounts of these two varying with the species of animal. These figures for tiger and puma fat appear to provide an example of the effect of diet on fat composition, for the fats previously examined come from animals in their natural habitat and feeding mainly on deer and sheep both of which produce 'stearic-rich' fats. The fats now examined, on the other hand, come from animals fed partly on horse flesh and the composition of their fats shows some resemblance to horse fat which is not stearic-rich, but highly unsaturated with octadecatrienoic acid as a major component acid. These figures are collected together in Table 6, the values for stearic and the polyethenoid C_{18} acids being particularly illustrative.

The results now available suggest that the fat laid down by carnivorous animals contains a high proportion of assimilated fat, so that the composition will be highly dependent on the diet. In particular, captive animals fed on horse flesh will produce fat quite different from animals living on species which produce 'stearic-rich' depot fats.

SUMMARY

1. Specimens of fat from a tiger and a puma kept in captivity have been examined. They are more unsaturated than specimens previously reported from wild animals of the same species.

2. These differences are probably related to differences of diet.

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The Reaction Between Elastase and Elastic Tissue

1. THE SUBSTRATE

By D. A. HALL*
Department of Medicine, University of Leeds

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The attempted characterization of elastin by observations based on any single property was first criticized some time ago (Hall, 1951). Since then, as part of a co-ordinated project, studies of elastic tissue have been made by both histological and electron-microscopical techniques (Hall, Reed & Tunbridge, 1955); further examination of such tissue has been made by X-ray diffraction methods and preliminary chemical and biochemical observations on the enzymic degradation of elastic fibres have also been reported (Hall, Reed & Tunbridge, 1952; Hall, 1952, 1953). As part of a study of the role of elastin in connective tissue, measurements have been made of those physical properties associated with the physiological function and mechanical stability of the fibres (Wood, 1953, 1954).

Many of these observations have been made on tissue which had been treated with acetic acid to remove the associated collagen (cf. Gross, 1949). Other groups of workers have described different methods for the removal of collagen, employing urea (Stein & Miller, 1938) or alkali (Banga & Schuler, 1953; Lansing, Rosenthal, Alex & Dempsey, 1952). Recently, however, Banga (1953) has put forward the suggestion, based on the observations of Schwarz & Dettmer (1953), that elastin is drastically altered, especially with respect to its internal structure, when treated with such solutions at 100°, during the removal of collagen. In view of this, study of the substrate and the possible effect of the various pretreatments which it may undergo was undertaken before embarking on study of the reaction between the enzyme and substrate. Similarly, it has been considered advisable to keep an open mind regarding the identity of 'elastin'. For this reason, the materials examined are to be regarded as elastic-tissue preparations, the term elastin being used simply as a convenient abbreviation.

MATERIALS AND METHODS

Elastic tissue. Preparations from three sources have been employed: human aorta, ox aorta and ox ligamentum nuchae. The ox tissues were obtained from freshly slaughtered young beef cattle; otherwise the method of preparation was identical with that described below for human aorta.

Segments of aortic arch were collected at autopsy. Although the age range of the aortae was considerable (20–65 years), only those showing no gross signs of atheromatous degeneration were used. The appearance of even one or two cholesterol plaques beneath the intimal membrane was regarded as sufficient to disqualify the aorta from inclusion in the pool. The accepted tissue was freed from adventitia and the intima stripped away. The remaining media was cut into 5 mm. squares and minced in a power-driven mincer. Stack (1950) has shown that, owing to heating, considerable degradation of connective-tissue proteins can occur during such preparative procedures. To obviate this, the aorta was chilled to $-69°$ with solid CO_2 before and between passages through successively smaller cutting plates. The mince was suspended in 4 vol. of 5% (w/v) NaCl solution and the maceration continued in a top-drive blender. Here again the jar and its contents were thoroughly chilled at frequent intervals during the operation. After filtration and thorough washing, a dried powder was obtained either by freeze-drying or by dehydration with acetone. Excessive drying of these powders might be expected to alter their properties, especially with respect to their resistance to enzymes, and they were therefore kept at 18° over sat. NaCl solution giving relative humidity 75%. The various treatments described below, with the exception of that with urea, did not markedly affect the water regain of the powders, which had an average value of 14·5%. For convenience, therefore, the weights recorded are for tissues equilibrated in this fashion and are referred to as 'air dry'. In the case of the material treated with urea, there was evidence from nitrogen determinations that appreciable amounts of urea remained attached to the protein even after exhaustive washing, and this greatly increased the water uptake.

Considerable difficulty has arisen in the study of the action of elastase on elastic-tissue preparations from various sources, on account of the impossibility of grinding the tissue to uniform particle size, without considerable denaturation due to heating. Fractionation of samples according to size could concentrate tissue components of different types in different size fractions, especially in the case of aorta, where there is evidence of at least two types of elastin-staining element (Benninghoff, 1927). Ground ox aorta was therefore fractionated in standard sieves and treated with elastase. The figures in Table 1 show that, although all the samples have very similar susceptibility to the enzyme, there is a slight increase in activity as the size of particle decreases. This may merely be due to different rates of penetration into particles of varying size, but in order to

* Nuffield Gerontological Research Fellow.

ensure comparable results sets of experiments were confined to one size range, and where possible the smaller sized material was employed. This was considered advisable in view of the observation that in the larger size ranges some of the pieces were compact and dense while others were fibrous and light. These two fractions could be separated in a similar fashion to that suggested by Lansing, Roberts, Ramasarma, Rosenthal & Alex (1951) and the enzyme was 34% more active on the fibrous material than on the dense substance.

Elastase preparations. Elastase was obtained by a method which differed only slightly from the original procedure of Balo & Banga (1950). The small amount of fractionation carried out, however, did appear to have a purifying effect. Dried defatted commercial hog pancreas powder (Viobin, Inc. Montecello, Illinois, U.S.A.) was extracted twice with 5 vol. 0·1M sodium acetate buffer (pH 4·7). Two other commercial samples of pancreas powder (Armour Laboratories, London) were found to be considerably less active as sources of elastase either as bought or after exhaustive solvent extraction. The bulked extracts were brought to 45% ammonium sulphate concentration (w/v) and the precipitate was collected. The precipitate was dissolved in a minimum quantity of water and dialysed in cellophan against running tap water for 24 hr. and then against two changes of 500 vol. of dist. water. A precipitate formed during dialysis, which could be redissolved on the addition of NaCl to 0·5–1% (w/v). The precipitate, carefully dried with acetone at 0°, was used as the typical enzyme preparation. The pH/activity curve for the enzyme was of a similar shape to that reported by Balo & Banga (1950), having a shoulder at pH values below that of the peak.

Protein determination. Hydrolysis was carried out by boiling under reflux in 5 vol. of 5N-HCl for 24 hr. HCl was removed by treatment on columns of Zeo-Karb 226 (Permutit Co. Ltd., London) according to Piez, Tooper & Fosdick (1952). Two-way filter-paper chromatograms were made by the ascending technique on 25 cm. squares. The two solvent systems were *n*-butanol–acetic acid–water (50:10:40, by vol.) and *n*-butanol–pyridine–water (50:10:40, by vol.) adapted from Evans, Evans & Parr (1949). Figures relating to the concentration of the amino acids were obtained by visual assessment of the ninhydrin colours on comparison with known amounts of individual amino acids which had been subjected to the same chromatographic procedure. Protein determinations on the enzyme solutions were made by the method of Lowry, Rosebrough, Farr & Randall (1951), employing a mixed biuret and Folin & Ciocalteau reagent. In the biuret solution the tartrate was replaced by glycerol to facilitate solution of the copper.

Table 1. *The effect of particle size on the activity of elastase on elastin preparations from ox aorta*

Air-dry ox-aorta elastin (50 mg.) suspended in 10 ml. 0·1M sodium glycinate buffer (pH 9·0) 0·4M in respect of NaCl and incubated at 37° for 17 hr. with 0·5 mg. elastase.

Particle size (mm.)	Percentage dissolved
Above 2·5	68·1
1·2–2·5	68·0
0·8–1·2	69·1
0·6–0·8	72·7
Below 0·6	74·6

Protein determinations on the supernatant after the enzyme had been allowed to react were carried out using the biuret reagent of Sols (1947). Reagent (3 ml.) and water (2 ml.) were mixed with 2 ml. of the supernatant. The absorption was read after 15 min. without a filter in the EEL. photometer (Evans Electroselenium Ltd., Harlow, Essex). A reading of 25 scale divisions was equivalent to the complete solution of 50 mg. of air-dry protein and the relationship between colour value and protein concentration in the supernatant was linear over the whole of this range.

Polysaccharide determinations. Hexosamine was determined by the method of Boas (1953) on hydrolysates prepared by 6 hr. treatment with 5N-HCl under reflux, and are expressed in terms of D-glucosamine, which was used as standard.

The total reducing substance present in the preparations was determined using the Hagedorn & Jensen method, on the same hydrolysates. Results are expressed as mg. glucose/g. air-dry tissue.

Sulphate was determined using the method of Lugg (1938) for total sulphur, since the non-sulphate sulphur was of negligible amount.

Estimation of the reaction between enzyme and substrate. Except where otherwise stated, 50 mg. of elastin were used in all the measurements of enzyme activity. This amount of elastin was suspended in 9 ml. of buffer in a 25 ml. Erlenmayer flask. The various effects with different buffers are described by Hall & Gardiner (1955). In the majority of the experiments, the buffer was either 0·1M sodium glycinate in 0·2M-NaCl or 0·1M sodium barbiturate, both at pH 8·7. The reaction was carried out in flasks because the reproducibility of results was dependent on there being a sufficiently large surface area of buffer for the tissue powder to lie in a single layer. To the reaction vessel a solution of enzyme preparation (50–1000 μg. in 1 ml. physiological saline) was added. Since it was observed that shaking may cause inactivation of the enzyme, the mixtures were generally incubated at 37° for 18 hr. without shaking. After incubation, the flasks were immersed in boiling water for 3 min. to destroy the enzyme, the residue was centrifuged down, and the activity measured either by drying and weighing the residue, or more easily by measuring the soluble-protein content of the supernatant.

RESULTS

Treatment with acetic acid

Ligamentum nuchae powder was autoclaved in 50 vol. of 1% (w/v) aqueous acetic acid for 2 hr. at 15 lb./sq.in. In Table 2 are given figures relating to the activity of the enzyme when tested against this substrate. For comparison a similar set of figures are given for the same enzyme on the untreated whole ligament. From these it can be seen that elastase is 2–4 times more active on acetic acid-treated material than on whole tissue. Similar effects can be observed after treatment with acetic acid under a number of conditions, all of which are adequate for the removal of the structurally discernible collagen, as judged by electron-microscopical examination. They range in severity from

boiling with frequent changes of 10 vol. of 1 % acetic acid for periods of up to 5 days, to shaking at 37° for 24 hr. with 50 vol. of 2 % acetic acid. Histological examination of tissue treated by these methods showed, however, that an appreciable amount of amorphous material staining as collagen still remained, especially in human aorta. Relative amino acid analyses for human aorta and ox ligament, both treated with acetic acid, are given in Table 3, and can be compared with similar values calculated from the microbiological assay figures obtained by Neuman (1949) for ligament elastin.

Treatment with urea solution

Stein & Miller (1938) claimed that their preparation of elastin from ox ligamentum nuchae was resistant to solution in boiling 40 % urea solution for periods of up to 5 days. As has been reported earlier (Hall, 1951), this resistance is relative, being a function of the urea/tissue ratio. A period of between 90 and 100 hr. at 100° was required at urea levels above the threshold value for the elastin to pass into solution. With shorter periods of treatment, only small amounts of protein dissolved. Table 4 records the degree of dissolution which occurs with time and the susceptibility of the residue to elastase attack, while the changes in amino acid constitution which occur as urea treatment proceeds can be followed from Table 5.

During urea treatment the pH of the solution rose to a value of 9·8 and ammonia was liberated. The concentration of urea was so large, however, that it was impossible to tell by urea analyses, whether the ammonia came from the breakdown of the urea or from the tissue itself.

Table 2. *The effect of acetic acid treatment on the susceptibility of a ligament preparation to attack by elastase*

Acetic acid treatment as follows: powdered ox ligament (0·6–0·8 mm. particle size) autoclaved for 2 hr. in 50 vol. 1 % (w/v) acetic acid at 15 lb./sq.in. pressure. The conditions of the enzyme reaction were otherwise as in Table 1.

Elastase μg./ml.	Photometer readings (see Methods) Whole tissue	Acetic acid-treated
5	1·5	7·7
10	2·0	9·3
15	2·4	12·0
20	3·2	13·6
25	4·8	14·0
30	5·2	14·4

Treatment with alkali

The increased susceptibility of elastin which had been treated with urea to elastase attack, and the fact that Banga, Balo & Nowotny (1949) reported an increase in elastase activity of 200 % after alkali treatment of the substrate, could indicate that the substrate was purified during alkali treatment. It has, however, been shown that it is possible to dissolve elastic tissue in 0·1 N-NaOH if the solid is boiled in the alkali for 6 hr. under reflux (Hall *et al.* 1952). Degradation might therefore also occur while the purification proceeded.

Elastic-tissue preparations, which had had the collagen removed by autoclaving with acetic acid as described above, were employed as starting materials in experiments aimed at studying the

Table 3. *Amino acid composition of various elastin preparations*

The figures in columns 2, 3 and 5 were obtained by visual assessment of the ninhydrin colours, those in column 4 by calculation from the microbiological-assay values of Neuman (1949).

Amino acid	Arbitrary units proportional to amino acid concentration Whole human aorta	Acetic acid-treated aorta (autoclaved)	Ligament elastin (Neuman, 1949)	Ox ligament acetic acid-treated (autoclaved)
Alanine	10	10	8	10
Arginine	6	3	—	0
Aspartic acid	6	2	—	1
Glutamic acid	9	5	—	2
Glycine	10	9	10	10
Hydroxyproline	3	2	—	0
Isoleucine	4	3	4	6
Leucine	4	3	6	5
Lysine	8	4	1	3
Methionine	5	1	—	0
Phenylalanine	3	2	5	3
Proline	7	6	8	6
Serine	7	1	2	0
Threonine	4	0	2	0
Tyrosine	2	2	3	1
Valine	7	6	8	7

Table 4. *Loss of weight of elastin preparations at various stages during a purification procedure, and the effect of elastase on them*

The percentages in column 2 are calculated on the original air-dry weight of the tissue. Those in column 3 on the air-dry weight of the individual preparation.

Tissue	Loss in weight during treatment (%)	Loss in weight after treatment with elastase (%)
(1) Whole human aorta	0	46
(2) 1 freed from collagen by acetic acid	16	12
(3) 2 after 24 hr. treatment with 40% urea	28	16
(4) 2 after 48 hr. treatment with 40% urea	43	43
(5) 2 after 72 hr. treatment with 40% urea	60	96·5

Table 5. *Amino acid composition of residues after urea treatment*

Values for amino acid content determined as for Table 3. Because of the retention of urea in the residues after urea treatment, and hence the differing moisture content, the hydrolysates were diluted to equal amino acid content, instead of being compared at similar air-dry weights.

	Arbitrary units proportional to amino acid concentration				
		Days treatment with urea			
Amino acid	Collagen-free aorta	1	2	3	4
Alanine	10	10	9	9	8
Arginine	3	1	0	0	0
Aspartic acid	2	2	1	1	0
Glutamic acid	5	4	2	3	2
Glycine	9	10	8	8	7
Hydroxyproline	2	1	0	0	0
Isoleucine	3	3	1	2	2
Leucine	3	3	2	3	2
Lysine	4	1	1	1	1
Methionine	1	0	0	0	0
Phenylalanine	2	3	1	2	1
Proline	6	8	6	7	6
Serine	1	0	0	0	0
Tyrosine	2	2	1	1	1
Valine	6	8	7	6	6

effect of the period of alkali treatment on the substrate. After autoclaving, the powder was extracted with water until the extract no longer gave a biuret reaction even after tenfold concentration. The resulting powder had the following characteristics expressed in mg./g. of air-dry material: N, 141; SO_4^{2-}, 4·65; moisture, 147; ash, 6·7; polysaccharide, as glucose, 4·2.

Determination of the effect of elastase on a series of elastin preparations which have had various periods of alkali treatment depended on the accurate timing of the process. This was accomplished as follows. A volume of 0·1 N-NaOH such as to give a solid:NaOH ratio of 1:20 was boiled under reflux. Momentary removal of the condenser was followed by the introduction of the solid down a polythene chute so that it became rapidly wetted. The solution was returned to the boil within 1 min. and the reaction timed from the recommencement of boiling. At the end of the required period the whole mixture was poured into 500 vol. of ice-cold water, thus completely arresting the reaction. The residues were then thoroughly washed, dried with acetone and allowed to equilibrate over saturated NaCl solution.

The properties of a series of alkali-treated preparations

A series of alkali-treated substrates was prepared by the method described above, differing only in time of treatment, which increased progressively by 20 min. intervals to 4 hr. Chemical characterization of these included protein, amino acid and polysaccharide and sulphate determinations.

Amino acid composition. Chromatograms of hydrolysates of all the preparations showed no discernible differences in amino acid composition as the period of treatment increased. In all cases the values were similar to those given in Table 3.

Polysaccharide. Determinations of total reducing substance showed a progressive fall from a value of

0·4 % during the first 80 min. alkali treatment. Thereafter the content was very low, approx. 0·02 % and fluctuated slightly. The hexosamine values are given in Fig. 1. The sulphate content of the preparations dropped within 1 hr. to 40 % of its initial value, and after 2 hr. remained constant at values below 23 % of this initial figure. The effect of elastase on the alkali-treated substrates was also examined. The results of a typical experiment are given in Fig. 2. From this curve it can be seen that as alkali treatment proceeds, the action of the enzyme at first increases and then falls rapidly. The degree of alkali treatment required for peak action of the enzyme was not always the same for different batches of elastase, but always lay within the range 0–60 min. The sharp drop in activity which occurs after alkali treatment has been permitted to proceed beyond the optimum length of time, is however, not permanent, since if alkali treatment is prolonged beyond 80 min. the curve relating activity to alkali treatment rises again.

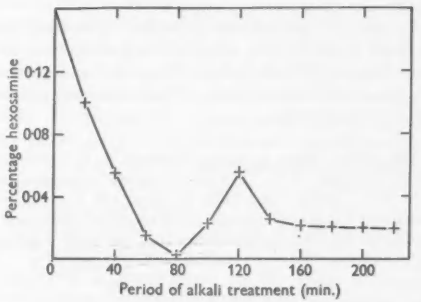

Fig. 1. The removal of hexosamine by alkali treatment. The hexosamine content is expressed as a percentage of the air-dry weight of the individual alkali-treated elastic tissue preparations (calculated as glucosamine).

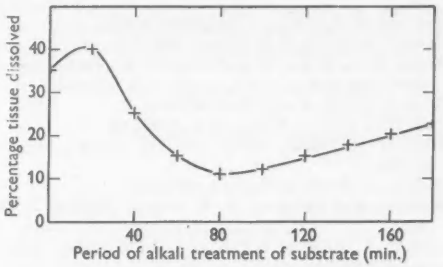

Fig. 2. The effect of elastase on a series of alkali-treated substrates. The substrates were boiled with 0·1 N-NaOH for the periods stated. The elastase was allowed to react with 50 mg. of each in 10 ml. 0·1 M-Na glycinate buffer for 17 hr. at 37° at a concentration of 25 μg./ml.

DISCUSSION

One of the main difficulties in assessing the value of analytical work on elastin, as that on other fibrous proteins, is uncertainty as to whether the material has been adequately freed from associated proteins without having been degraded itself. All the chemical methods which have been suggested for the removal of collagen employ heat and require the presence of moderately strong acid or alkali.

Boiling with 1 % acetic acid, which does not itself appear to be adequate for the removal of all the 'non-elastin protein' from human aorta must be assumed to have some far-reaching effect since there is a 200 % increase in the action of elastase tested against this substrate in comparison with a substrate prepared from whole ox ligament. In the same way, treatment with boiling 0·1 N-NaOH must produce a marked change in the substrate, since over the first 90 min. boiling, there is first an increase in susceptibility to elastase, followed by a fall to a very low value. The possibility exists that the residues after acetic acid extraction of human aorta and ox ligament represent two elastins of quite dissimilar amino acid composition. Further treatment of acetic acid-extracted aorta with boiling 40 % (w/v) urea solution, however, causes a marked drop in the concentration of polar amino acids and of hydroxyproline, leaving a residue which has an amino acid analysis closer to that of ox ligament which has been treated with acetic acid alone. The removal of these particular amino acids would indicate that the aortic residue after acetic acid treatment contained a fraction similar to collagen in composition but differing from it in its solubility properties. It is interesting to note that Lansing *et al.* (1951) have reported an increase in just these amino acids in human aortic-elastin preparations as the age of the sample increases. Another explanation of the difference between the acetic acid residue from aorta and ligament arises from a consideration of observations made on these tissues by electron-microscopic techniques (Hall *et al.* 1955). In its simplest form, in ox ligament, elastic tissue appears under the electron microscope to be composed mainly of cylindrical fibres which are opaque to the electron beam. If these fibres are treated with elastase the opaque layer is removed, and the fibres no longer appear cylindrical. At the same time an internal fibrillar structure is revealed. This, however, also is susceptible to attack by the enzyme, and ultimately the whole of the tissue passes into solution. Aorta behaves similarly, except that, especially in the lamellar areas of the aortic elastin (Benninghoff, 1927), the fibres do not appear as simple structures of fibrous nature, but as fibres embedded in an amorphous matrix which makes up the body of the lamella. This material is

opaque and is the first part of the tissue to dissolve under the action of elastase, revealing a fibrillar inner structure, as with ligament. The suggestion that 'elastin' consisted of two components has been made before (Hall *et al.* 1952), but the results now reported enable the hypothesis to be expanded. The elastic-tissue preparations from the two sources appear to differ from one another only in the relative concentrations of the amorphous and fibrillar components. Analyses of the alkali-treated substrates can give some indication of the way in which these two components differ from one another. As can be seen from Fig. 2, treatment with alkali for periods of up to 1·5 hr. causes a steady drop in the amount of hexosamine present in the preparation to a minimum value. This minimum value for hexosamine and hence by inference, for polysaccharide, coincides with the period of alkali-treatment which is required for the enzyme susceptibility of the substrate also to reach a minimum. It therefore appears likely that during this period of treatment a fraction of the tissue containing most, but not all, of the polysaccharide present was being dissolved. In the earlier paper in which tentative suggestions regarding the structure of elastin were proposed (Hall *et al.* 1952), it was reported that the whole of the acid liberated by the enzyme was freed during the dissolution of the first half of the protein. It was therefore assumed that the portion of the elastin which was dissolved during this early part of the reaction contained the whole of the polysaccharide. Correlation of elastase susceptibility and the hexosamine content of the alkali-treated substrates indicates that the polysaccharide is not confined to one of the two components of elastic tissue.

A study of the substrate of the elastin–elastase system therefore reveals the following facts. The elastic fibres, as defined by histologists or electron-microscopists, namely the fine fibres and lamellae in aorta and the thick fibres of ligament, do not consist of a single entity. Preparations which have been obtained for chemical studies by alkali or urea treatment are most likely to have had the material containing the majority of the polysaccharide removed. The properties of these preparations will approach those of the whole elastic fibre only in the case of ligament. The relative proportions of the two components will cause the fibrillar material to play a more important role in determining the properties of 'elastin' preparations from this source. All methods at present available for the separation of two components, such as the removal of an amorphous outer layer, are so drastic that it must be assumed that degradation of the fibrils occurs simultaneously. For this reason it will be observed that a series of substrates which have had different periods of alkali treatment has been employed in the enzyme studies reported in this and the following paper (Hall & Gardiner, 1955). No claim is made for the identity of any particular substrate, and the name elastin is not applicable to a particular entity which can be identified by biochemical and histological methods.

SUMMARY

1. Various methods have been examined for the isolation of an entity, to which the name 'elastin' can be applied.

2. They have been compared by observing the changes produced in the susceptibility of the resulting material to attack by elastase.

3. It is suggested that all existing methods for freeing so-called elastin from associated proteins are either inadequate or so drastic as to commence the degradation of the fundamental unit of elastic tissue itself.

4. For a study of elastic tissue, a series of alkali-treated preparations appear to offer the greatest scope.

5. Further evidence is provided indicating that at least some of the 'elastin' preparations which have been described consist of more than one component, but that in many of these the proportions differ considerably.

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The Reaction Between Elastase and Elastic Tissue

2. PREPARATION AND PROPERTIES OF THE ENZYME

By D. A. HALL* and J. E. GARDINER
Department of Medicine, University of Leeds

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Balo & Banga (1949*a*) first described an enzyme in pancreas capable of causing the total solution of elastin preparations from aorta. Earlier observations on the bacterial digestion of connective tissue (e.g. Ewald, 1890) had demonstrated that enzymes with similar properties were present in a number of micro-organisms. The cultures employed were impure, however, and no attempts were made to isolate the enzymes concerned. Balo & Banga (1950) described the extraction and partial purification of an enzyme from pancreas and suggested the name elastase. Recently, preparations obtained by their method have been employed in attempts to elucidate the structure of elastin (Banga & Schuler, 1953; Lansing, Rosenthal, Alex & Dempsey, 1952; Hall, Reed & Tunbridge, 1952). Balo & Banga (1949*b*) have also shown that there may be a connexion between the activity of elastase and the onset of arteriosclerosis.

MATERIALS AND METHODS

Substrates and sources of enzyme preparations were the same as those used in the preceding paper (Hall, 1955). The active enzyme used in the experiments described below had similar properties to samples kindly supplied by Professor I. Banga, whom the authors would like to thank both for these gifts and for permitting them to see, before publication, a copy of a paper in which she described in greater detail her method of purifying elastase.

Methods. These were essentially the same as those described in the preceding paper. Where purification of the enzyme permitted maximum solution of 50 mg. of substrate at very low enzyme concentrations, it has been found desirable to express the activity of the enzyme in terms of 'specific activity values'. Specific activity is calculated from the 'observed activity' i.e. the biuret value of the supernatant from a system containing 50 mg. of 'elastin' incubated for 17 hr. at 37° in 10 ml. of pH 8·7 buffer, so as to indicate the amount of soluble protein (in mg.) which would be produced by the action of 1 mg. of elastase.

RESULTS

Extraction procedures. The majority of the elastase preparations examined have been obtained by the extraction of defatted pancreas powder with 0·1 M sodium acetate buffer (pH 4·7) as suggested by Balo & Banga (1950). Pilot experiments have shown, however, that extraction at alkaline pH values is quite as effective in removing elastase from the tissue, but removes much inert material as well (Table 1).

* Nuffield Gerontological Research Fellow.

Table 1. *Activity of enzyme at various early stages in the extraction procedure*

Source of enzyme preparation		Specific activity value (see text)
Whole pancreas powder—dialysed		2·9
0·1 M Sodium acetate buffer (pH 4·7) extract—dialysed	Solution	3·0
	Precipitate	170·0
0·1 M Sodium phosphate buffer (pH 8·7) extract—dialysed	Solution	2·8
	Precipitate	79·4
45% $(NH_4)_2SO_4$ precipitate—dialysed	Solution	1·64
	Precipitate	182

Table 2. *Activity of various precipitates derived from fraction A (see text)*

The figures indicate the amount of soluble protein (mg.) obtained from 50 mg. elastin in 10 ml. 0·1 M sodium glycinate buffer pH 8·7.

Method of preparation of enzyme fraction	Enzyme added (μg.)/50 mg. elastin			
	50	100	200	500
Dialysis at 18° (*Pw*)	25·4	37·0	45·0	45·8
Dialysis at 0° (*Pc*)	20·6	34·0	42·0	46·4
Precipitation with acetone (*Pa*)	9·0	15·4	22·8	36·4
Whole 45% $(NH_4)_2SO_4$ precipitate dried with acetone (*Px*)	12·0	20·8	31·0	40·6

Table 3. *The effect of* pH *on the precipitation of elastase*

Treatment of enzyme preparation (see text)	Specific activity value (see text)
Fraction *Px*	160
Precipitate at pH 9·0	530
Solution at pH 9·0	1200
Precipitate at pH 6·0	212
Solution at pH 6·0	1010
Precipitate at pH 5·0	150
Solution at pH 5·0	370

Table 4. *Solution of elastin preparations under the influence of elastase*

Incubation 16 hr. at 37° 50 mg. elastin, 500 μg. elastase in 2·5 ml. 0·1 M sodium glycinate buffer (pH 10·3).

Source of elastin	Percentage dissolved
Human aortic media	86·5
Ox aortic media	45·0
Ox ligamentum nuchae	3·5

Precipitation with ammonium sulphate. Complete precipitation of the enzyme from acetate buffer can be accomplished by adding 45%, by weight, of ammonium sulphate (based on the initial volume of the buffer extract) (Table 1). This material (*A*) has been used as the starting point for the majority of the fractionation procedures described below. The moist precipitate from this stage was dissolved in the minimum quantity of distilled water and dialysed against running tap water for 24 hr. followed by two changes of 500 vol. of distilled water. During dialysis a precipitate formed and was collected and dried with acetone (*Pw*). A further precipitate could be obtained by chilling the contents of the dialysis bag to 0° (*Pc*) and a fine cloudy suspension appeared if the solution were cooled to −15°. This was too small to collect and test, and was combined with the final fraction (*Pa*) which could be precipitated on the addition of 4 vol. of ice-cold acetone. The properties of these various fractions and their relative activity at various enzyme/substrate ratios are given in Table 2.

There was no evidence of any separation of the enzyme into components with different properties by this type of treatment, and it was found possible to redissolve *Pw* by the addition of 1% (w/v) aqueous NaCl, as originally suggested by Balo & Banga (1950). *Pw*, *Pc* and *Pa* were therefore regarded as consisting of essentially similar material and in further experiments were bulked together to give *Px*.

Examination of dialysed fraction Px. The whole dialysed mixture containing both precipitate and solution was brought to pH 9·0 with 2 N-NH_3 and centrifuged. The soluble portion was brought to pH 5·9–6·0 with 10% (v/v) acetic acid and a further precipitate removed by centrifuging, while a final precipitate was obtained on the addition of more acetic acid to pH 5·0. The precipitate obtained at pH 9·0 was washed free of any remaining solution with one-third of the original vol. of 0·02 N-NH_3. The specific activity values of these various fractions are given in Table 3. From this it can be seen that although some active material remains undissolved at pH 9·0, the greater part passes into solution and is still in solution at pH 6·0; however, on bringing the pH to 5·0 there is a marked loss in activity. The solubility of the enzyme, especially at pH 9·0, is dependent on the ionic strength of the solution. Here again separation of enzyme from inactive material appeared to take place, but the enzyme did not appear to contain more than one component.

Sensitivity to pH *of the enzyme–substrate system.* Balo & Banga (1950) published data for pH/activity curves for the enzyme acting on elastic-tissue preparations from aorta. Repetition of their experiments, using, however, sodium glycinate buffer, led to exactly the same results with human aortic material. From these observations it appeared that use of 2·5 ml. of 0·1 N buffer (pH 10·3) was optimum for the dissolution of 50 mg. of elastic tissue under the action of up to 1 mg. of elastase. When attempts were made to treat ox ligament preparations in the same way, it was found that this substrate was completely refractory (Table 4). At lower pH values, however, ox ligament would pass into solution under the action of elastase. A comparison of the pH/activity relationships of the enzyme acting on the two substrates can be made from columns 1, 3 and 5 of Table 5.

Table 5. *Activity of elastase preparations at various* pH *values*

50 mg. elastin. 250 μg. elastase preparation. 2·5 ml. 0·1 M sodium glycinate buffer incubated for 16 hr. at 37°.

	Ox ligament		Human aorta	
Initial pH	Final pH	% dissolved	Final pH	% dissolved
8·15	8·04	25·3	7·37	31
8·52	8·11	37·2	7·37	31·3
8·74	8·45	49·05	7·51	36·6
8·99	8·51	59·6	7·75	45·3
9·29	8·62	66·0	8·05	51·2
9·57	8·75	72·65	8·34	64·0
9·81	8·83	68·5	8·50	72·1
10·03	8·97	46·25	8·71	79·1
10·31	9·55	23·73	8·93	81·2
10·47	9·83	3·93	9·01	84·5
10·60	10·27	4·25	9·05	81·9
10·80	10·57	3·34	9·07	81·0

Table 6. *Acid (m-moles/g.) liberated by elastase from elastin*

Conditions similar to those given in Table 5.

	Elastin	
Initial pH	Ox ligament	Human aorta
8·15	0·09	0·08
8·52	0·09	0·08
8·74	0·19	0·19
8·99	0·25	0·25
9·29	0·36	0·37
9·57	0·55	0·54
9·81	0·50	0·50
10·03	0·46	0·60
10·31	0·43	0·72
10·47	0·40	0·80
10·60	0·13	0·82
10·80	0·04	0·84

Although there appeared to be such a marked difference between the two enzyme–substrate systems, the supernatants from those systems which had been allowed to react under optimum conditions showed considerable similarity, in that the final pH of the buffer solution had fallen to the region of 8·5–9·0. The final figures corresponding to a series of initial pH values are given in the remaining columns of Table 5, and these indicate that large amounts of acid must have been liberated during the course of elastase action.

Increasing the buffer concentration so that the ionic strength of the buffering salt was increased fourfold, and at the same time increasing the volume of buffer to 10 ml., considerably reduced the fall in pH. Curves obtained in this way and showing the pH/activity relationship of the enzyme–substrate systems are identical with those which can be prepared from the figures in the last four columns of Table 5. It can be seen that although there are differences between the shapes of such curves, the optimum values fall between pH 8·7 and 9·0.

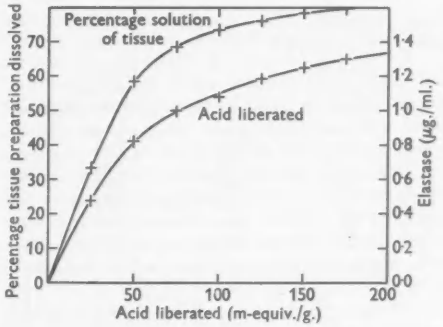

Fig. 1. Comparison of the acid liberated, and the protein dissolved during the action of varying amounts of elastase on a preparation from human aorta. With the exception of the amount of elastase, the conditions were identical in each case: 50 mg. of elastin. 2·5 ml. 0·1 M sodium glycinate buffer, pH 10·3, incubated for 17 hr. at 37°.

Nature, amount and distribution of the liberated acid. Human aorta preparations contained 7·9 mg. sulphate/g. air-dry protein, whereas ox ligament preparations contained 4·65 mg. This sulphate was measured as total sulphur (see preceding paper), but was identified as sulphate after hydrolysis, and in the supernatant after the action of the enzyme, by a variety of procedures. Reaction with $BaCl_2$ in the presence of $KMnO_4$ produced a pink precipitate which could not be decolorized with $K_2C_2O_4$. Solutions also gave a heavy precipitate with benzidine. Whether these observations indicate the liberation of free sulphuric acid or of a sulphate radical still bound to sugar residues, cannot be decided yet, but the amounts liberated from aorta and ligment can be measured by titration of the buffer solution. Fig. 1 demonstrates the relationship between the liberation of acid and the amount of protein passing into solution under the action of

differing amounts of elastase. A comparison of the amounts of acid released from aorta and ligament preparations at various pH values can be obtained from the figures in Table 6. It appears that at initial pH values of above 9·8 there is a rapidly decreasing amount of acid liberated from the substrate in the case of the ligament, whereas the acid liberated from the aorta preparation continues to increase in amount. There is also a corresponding difference in the amount of protein passing into solution over this pH range.

As the tissue passes into solution under the action of elastase, acid is released, but as can be seen from Table 7, the change in pH accompanying the release of acid is complete after the dissolution of only half of the protein.

Table 7. *Release of acid with time of incubation*

250 μg. elastase. 50 mg. elastin. 2·5 ml. pH 9·57 0·1 M sodium glycinate buffer.

Time of incubation (hr.)	Final pH	Amount dissolved (% of sample)
1	8·99	10·45
2	9·02	21·6
4	8·82	23·2
6	8·63	43·6
8	8·33	56·1
12	8·35	72·0
17	8·34	89·1

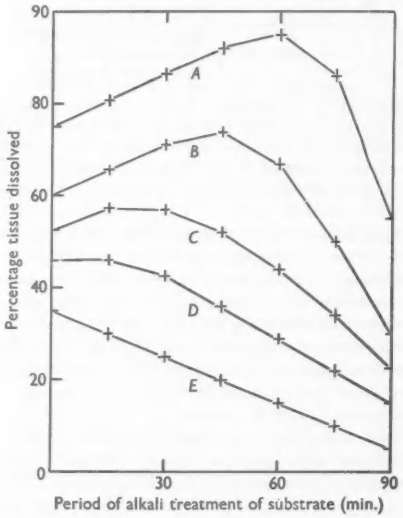

Fig. 2. The effect of alkali treatment on the susceptibility of ox ligament elastic-tissue preparations to attack by elastase. In all cases 50 mg. of each substrate were allowed to react at 37° for 17 hr. with the enzyme in 0·1 M sodium glycinate buffer (pH, 8·7). The concentrations of elastase were as follows: *A*, 100 μg./ml.; *B*, 80; *C*, 60; *D*, 40; *E*, 20.

The activity of the enzyme on alkali-treated substrates. In the preceding paper (Hall, 1955) it is reported that treatment of the collagen-free substrate with boiling alkali causes a marked change in its susceptibility to elastase. In Fig. 2 are given results of a typical series of experiments in which the amount of elastase employed was altered as well as the type of substrate. At concentrations of enzyme lower than those usually employed (250 μg./50 mg. tissue), the peak activity which was reported in the previous paper moves to shorter periods of alkali treatment, whereas at concentration of enzyme above the normal level, the peak moves to periods of longer treatment. The fall to minimum activity occurs at 80–90 min., however, in all cases, and the rise which was previously observed after longer periods in alkali takes place at a constant rate at all elastase concentrations.

The effect of alkali pretreatment of the substrate on the pH *sensitivity of the enzyme.* Examination of the effect of pH on the activity of the enzyme when tested against a series of alkali treated substrates shows an effect of considerable importance. Banga, Balo & Nowotny (1949) showed that alkali treatment caused an increase in the amount of protein dissolved from tissue by elastase. It can be seen from the curves in Fig. 3 that, as alkali treatment

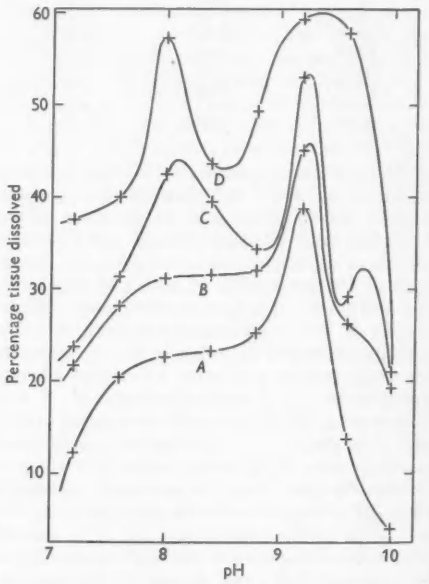

Fig. 3. pH/activity curves for elastase acting on a series of alkali-treated substrates. The substrates concerned have had the following periods (in min.) of alkali treatment: *A*, 0; *B*, 40; *C*, 120; *D*, 240.

Table 8. *The effect of varying the buffering salt on the activity of an elastase preparation*

The buffers were in all cases 0·1 M adapted from Vogel (1943) pH 7·8. The other conditions were as in Table 2, except that 250 μg. of elastase were used.

Period of alkali treatment of substrate (min.) ...	0	60	140	240
Buffer	Absorptiometer readings (see preceding paper)			
Na_2HPO_4–citric acid	1·5	4·5	4·3	7·0
KH_2PO_4–NaOH	3·7	10·2	8·6	12·0
Sodium diethyl barbiturate–HCl	7·4	9·3	7·2	13·3
Sodium glycinate–NaCl	6·6	11·1	5·0	14·4
H_3BO_4–NaOH–NaCl	12·3	13·6	7·8	14·6

proceeds, the degree of activity at the maximum around pH 9·0 increases. The more interesting fact is, however, that the shoulder which with untreated substrates is present in the pH range 7·0–8·0 becomes transformed into a second peak, the height of which also rises with alkali treatment.

The effect of buffering salts on the activity of the enzyme. The main peak and the shoulder of the pH/activity curve do not both lie within the range of maximum buffering capacity of one single simple buffer solution. However, the second peak which appears when the substrate is treated with alkali, lies well within the range of a number of buffer solutions and on the edge of the effective range of the glycine–NaOH buffer which has been employed for most of these experiments. The figures given in Table 8 are activity values of a particular enzyme preparation when tested in various buffers at pH 7·8. From this it can be seen that whereas a number of buffer solutions have a similar effect on the enzyme–substrate reaction, borate buffer enhances the reaction while a buffer containing citrate lowers the activity markedly.

DISCUSSION

Balo & Banga in their early papers on elastase described a number of the properties and the optimum working pH. Since initially their work was wholly concerned with human aorta, they did not observe the apparent resistance of ligament preparations to elastase attack at pH values which appeared optimum for the system when employing human tissue as substrate. The material liberated from the elastin by the action of the enzymes may differ qualitatively from source to source; it is, however, quite apparent that the acid liberated differs quantitatively. Above pH 9·5 the amount of acid liberated from ligament decreases rapidly, whereas that obtainable from human aorta and to a lesser extent from ox aorta continues to rise. Hence there is a marked drop in pH in the case of aorta but not in the case of ligament, at these high pH values. When the enzyme and substrate are suspended in a buffer solution of high pH but low buffering capacity, therefore, there is considerable drop in pH during the reaction with aorta but not with ligament. The pH is lowered sufficiently in the first case to approach the natural optimum of the system, but in the case of the ligament this value is not reached. When adequate buffering salts are present or when the final pH of the system is taken as the operative one, the true optimum of the system is seen to lie in the range 8·7–9·2 and not at 10·3 as stated by Balo & Banga.

When various concentrations of elastase were employed for the reaction with the alkali-treated substrates, the peak of optimum activity moved from zero to a period of 60 min. alkali treatment as the concentration of enzyme was increased. It is known that during this period of alkali treatment constitutional changes take place in the substrate, and it is conceivable that the relative concentrations of two substrates may alter. The movement of the peak could then be explained on the assumption that two enzyme systems exist which are specific for the two substrates, and that as the total enzyme/substrate ratio is altered the system consisting of alkali-treated substrate and its specific enzyme approaches its optimum working conditions. The movement of the peak would then indicate the resultant activity of the two enzymes, one already at its optimum, the other steadily increasing. The rise in activity which occurs after 90 min. must be assumed to be due to main chain degradation owing to hydrolysis of peptide bonds. The linkages which are susceptible to elastase attack are not affected since although the activity rises, the blank, i.e. the degree of solution in buffer alone, is not affected.

Further evidence in favour of there being two elastin components in elastic tissue and two specific elastases, arises from the pH/activity curves. The presence of a shoulder in the original curve of Balo & Banga was always suggestive of the presence of more than one enzyme. When, however, the release of the necessary substrate by the action of alkali causes the shoulder to change into a definite peak, there ceases to be any doubt. The second enzyme, which is the more active on the alkali-treated material, has a range of optimum activity of pH 7·8 to 8·1.

SUMMARY

1. Revised curves relating activity of the enzyme to pH have been obtained for elastase, the optimum pH range for which has been shown to be 8·7–9·2.

2. Although, if adequately buffered, the enzyme is capable of dissolving elastic tissue from three sources at this pH equally efficiently, the products of the reaction are not universally the same.

3. Increasing amounts of sulphuric acid are released from aortic preparations especially above an initial pH of 9·8, smaller amounts are liberated from ox ligament.

4. Evidence is advanced that elastase consists of two enzymes which act on two components of an elastic tissue preparation which is itself dual in nature.

5. The optimum pH of the second enzyme which utilizes alkali-treated elastic tissue as substrate lies in the range 7·8–8·1.

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Observations on the Relationship Between Pteroylglutamic Acid and Nicotinamide Metabolism

By PREMA FATTERPAKER, URMILA MARFATIA and A. SREENIVASAN
Department of Chemical Technology, University of Bombay, India

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Slightly depressed growth and fatty livers have been reported in rats fed moderate amounts of nicotinamide, together with a low protein diet (Handler & Dann, 1942; Handler & Bernheim, 1943). The fatty livers were susceptible to the action of choline, while methionine was found to alleviate the growth inhibition. Since excess of nicotinamide is detoxicated and eliminated as urinary N^1-methylnicotinamide, the observed toxicity of nicotinamide at unphysiological levels of feeding was attributed to forced methylation and consequent depletion of labile methyl reserves (Handler, 1944). In agreement with this, Handler observed no signs of toxicity as a result of ingestion of large quantities of nicotinamide by young rabbits or guinea pigs, neither of which species excrete N^1-methylnicotinamide (Handler, 1944). Production of fatty livers by feeding excessive amounts of glycocyamine, cystine or homocystine have also been reported (du Vigneaud, Chandler, Moyer & Keppel, 1939; du Vigneaud, Chandler, Cohn & Brown, 1940; Stetten & Grail, 1942). More recently, it has been shown that, in the pyridine-fed rat, there is a decreased urinary excretion of creatine owing to a reduction of available methyl groups, pyridine being detoxicated as N-methylpyridinium hydroxide (Dinning, Keith, Parsons & Day, 1950).

The known mediation of pteroylglutamic acid in biological methylations including the methylation of nicotinamide (Fatterpaker, Marfatia & Sreenivasan, 1951, 1952*a*, 1954; Dietrich, Monson & Elvehjem, 1952) suggested the present studies on its influence on the extent of depletion of methyl reserves in rat livers consequent on ingestion of excessive amounts of nicotinamide. The studies included observations on a number of other substances and enzymes expected to be influenced by changes in pteroylglutamic acid or available labile methyl compounds. Pteroylglutamic acid deficiency was induced in the first set of experiments by dietary means, while in the second series aminopterin feeding was used. In the first series, additional groups with choline supplementation were included.

EXPERIMENTAL

Methods

Urinary excretions of creatine (Clark & Thompson, 1949), N^1-methylnicotinamide (Huff & Perlzweig, 1947) and total nicotinic acid (Swaminathan, 1946), liver N (Johnson, 1941),

methionine (Horn, Jones & Blum, 1946), choline (Horowitz & Beadle, 1943) and total (neutral) as well as phospholipids (Bloor, 1943) were determined by the methods cited.

Liver choline oxidase activity was followed according to the procedure of Dinning, Keith & Day (1949) using 1 mg./ml. of choline chloride as substrate and following oxygen uptake for 1 hr. Xanthine oxidase activities (Dhungat & Sreenivasan, 1954) were determined both in liver homogenates and for *in vitro* synthesis after incubation of slices for 3 hr. at 37° according to Dhungat & Sreenivasan (1952) in their medium 2. For determinations of enzyme activities, 1 ml. of liver homogenates prepared in 5 vol. of 0·01 M phosphate buffer pH 7·4 in a chilled all-glass homogenizer were employed.

Liver pantothenic acid was estimated according to Skeggs & Wright (1944). Coenzyme A activity was followed by determination of extent of sulphanilamide acetylation (Lipmann & Kaplan, 1948) and is expressed as μg. of sulphanilamide acetylated/g. of dry liver. Adrenaline was determined by modifications in the procedure of Ghosh, Deb & Banerjee (1951). Ascorbic acid, cysteine and glutathione were destroyed by treatment with 10% (w/v) $NaHCO_3$. Further treatment with 5% (w/v) NaOH destroyed adrenaline without affecting uric acid and ergothionine. The colour developed by Folin's tungstic acid reagent was measured at 660 mμ. Satisfactory values were obtained in recovery experiments. Allowance was made for any noradrenaline present by using the procedure of Euler & Hamberg, as adopted by D'Iorio & Plaut (1952); at pH 4, all adrenaline and only less than 10% of noradrenaline are destroyed by oxidation with I_2 while at pH 6 both are oxidized. Adrenaline was also tested for chromatographically, using Whatman no. 1 paper and acetone–benzyl alcohol–N-HCl (75:25:15, v/v) and spraying with ferric sulphate–ferricyamide solution. Adrenaline had an R_F value of 0·86, the value for noradrenaline being 0·77.

Animal experiments

Two sets of experiments were carried out. In the first one with simple pteroylglutamic acid deficiency, adult rats (Wistar strain, 100–120 g. body wt.) were reared on a pteroylglutamic acid-free, purified ration consisting of (g./100 g. diet) vitamin-free casein, 10; iodinated casein ('Protomone', Cerophyl Laboratories, Kansas City, Mo.), 0·5; sesame oil, 6; shark liver oil, 2; salt mixture (U.S.P. no. 2), 4; sucrose, 9·5; and maize starch, 68; with the following vitamin additions (in mg./kg. diet): thiamine hydrochloride, 6; pyridoxine hydrochloride, 6; calcium pantothenate, 20; riboflavin, 10; nicotinic acid, 30; biotin, 1; *p*-aminobenzoic acid, 100; choline chloride, 500; inositol, 500; vitamin K (Menadione, Merck), 10; α-tocopherol, 50; and vitamin B_{12} (Cobione, Merck), 0·15. The vitamin additions provided in the basal diet were considered adequate for the hyperthyroid condition. The animals were divided into six groups of four each, with due attention to details of weight, sex and litter, at the end of 5 weeks when leucocyte counts revealed definite signs of pteroylglutamic acid deficiency. The six groups were: (1) basal, pteroylglutamic acid-free, purified ration; (2) basal plus nicotinamide; (3) basal plus nicotinamide plus choline; (4), (5), and (6) same as (1), (2) and (3) respectively with pteroylglutamic acid (100 μg./day/animal intraperitoneally). Nicotinamide and choline were mixed in the diets at 0·5% levels. Urines were collected for 3 days after 3 weeks on the test diets; values reported are averages of pooled samples. The animals were killed after 32 and 38 days on the experimental diets.

In preliminary trials, it was found that total daily food consumption was unaffected at this level of nicotinamide supplementation. Observations on daily food consumption over a fortnight revealed that the female rats took 7·7–8·7 g. (average 8·4 g.) of food/day each and the male animals 10·4–11·1 g. (average 10·6 g.). There was no difference in food intake specifically on account of pteroylglutamic acid or nicotinamide supplementation. Growth was, however, impaired in pteroylglutamic acid deficiency and as a result of nicotinamide feeding. The results obtained in this series of experiments are given in Tables 1, 3 and 4.

In the second series groups of rats (120 g.) that had been fed the laboratory stock ration were used; the diet consisted of (g./100 g. diet) whole wheat flour, 59; casein, 5; whole milk powder, 4; shark liver oil, 2; defatted peanut meal, 13; brewer's yeast, 4; wheat bran, 4; dried fish meal, 3; sesame oil, 2; and salt mixture (U.S.P. no. 2), 4. Pteroylglutamic acid deficiency was induced by daily subcutaneous injections of 25 μg. of aminopterin for 6 days, and half this dose for a further 6 days. Nicotinamide (25 mg./day/animal) was given orally. Toxic symptoms (diarrhoea, swelling and bleeding around the paws, eyes and mouth) began to appear after 10–12 days. There were control groups on stock diet alone with and without nicotinamide supplementation. The animals were all killed on the 12th day. The results obtained are given in Tables 2 and 5.

All determinations were carried out in duplicate and the values reported are the averages for four animals in each group except for adrenaline where pooled adrenals from two animals were used for each assay.

RESULTS AND DISCUSSION

Weight of body organs. There was an increase in the liver weight to body weight ratio in pteroylglutamic acid deficiency (Table 1). This difference was somewhat more pronounced in the aminopterin-fed animals (Table 2) and was also seen in respect of kidney and spleen. Administration of nicotinamide resulted in larger liver weights in both series, the results being less noticeable in the pteroylglutamic acid supplemented groups. An observation of interest was the enlarged adrenals in the pteroylglutamic acid deficient or aminopterin-fed animals, especially in groups receiving nicotinamide. The changes in adrenaline are discussed later.

Keith, Broach, Warren, Day & Totter (1948) have reported on the ratio of chick liver weight to body weight being inversely related to the pteroylglutamic acid content of a purified diet and on the greatly enlarged condition of the gall bladders of the deficient birds. Similar observations on liver weight/unit body weight have been made by Williams, Sunde, Cravens & Elvehjem (1950). Induced dietary deficiencies evidently impose a stress on the animal organism, resulting in exhaustion, enlargement of glands and organs and hypofunction.

Urinary creatine and N^1*-methylnicotinamide.* The effect of pteroylglutamic acid (Fatterpaker *et al.* 1951, 1952*a*; Dietrich *et al.* 1952; Dinning & Day, 1949) is confirmed (Table 3). Increased methylation of the extra nicotinamide ingested could be seen and is much less in the pteroylglutamic acid deficient animals than in replete ones. Choline increased the excretion of N^1-methylnicotinamide in the deficient groups. Excretion of nicotinic acid bears the expected inverse relationship to N^1-methylnicotinamide excretion. Creatine excretion is not influenced in nicotinamide toxicity. Supplementation with both pteroylglutamic acid and choline enhances urinary creatine values.

Liver lipids. As reported by Handler (1944) and others, forced methylation as by the fed nicotinamide, causes increased liver lipids. There is also a definite decrease in phospholipids. The effect is considerably augmented in pteroylglutamic acid deficiency (Table 1) or in the aminopterin-fed groups (Table 5) and is corrected by choline feeding.

Liver nitrogen, choline and methionine. A slight reduction in liver nitrogen (Table 1) is observable as a result of nicotinamide toxicity as well as in pteroylglutamic acid deficiency. Choline supplementation apparently helps in mobilization of proteins in the deficient livers.

Liver levels of choline and methionine are lowered in pteroylglutamic acid deficiency (Fatterpaker *et al.* 1954). Nicotinamide feeding results in a small but significant further reduction in these compounds only in the deficient group not receiving choline. A favourable influence of choline in the pteroylglutamic acid fed group is probably attributable to the low level of protein (10% casein) in the basal diet.

Table 1. *Changes in liver weight and composition*

For details, see Table 3 and text. The animals were killed at the end of 5 weeks on the test diets. The results are averages for four independent samples ± S.E.M.

Diet	Liver weight (g./100 g. body wt.)	Liver nitrogen (mg./g. dry liver)	Total lipids (g./100 g. fresh liver)	Phospholipids (g./100 g. fresh liver)	Methionine (mg./g. fresh liver)	Choline (mg./g. fresh liver)
Basal (no pteroylglutamic acid)	4·68 ± 0·12	28·65 ± 0·70	3·83 ± 0·23	1·36 ± 0·07	4·5 ± 0·13	4·1 ± 0·16
Basal + nicotinamide	5·50 ± 0·55	26·65 ± 0·33	4·79 ± 0·19	1·13 ± 0·04	4·2 ± 0·11	3·8 ± 0·11
Basal + nicotinamide + choline	4·08 ± 0·38	32·29 ± 1·46	3·67 ± 0·27	1·27 ± 0·06	4·9 ± 0·14	4·3 ± 0·07
Basal + pteroylglutamic acid	4·30 ± 0·18	32·29 ± 0·50	3·29 ± 0·14	2·08 ± 0·20	5·7 ± 0·20	4·8 ± 0·15
Basal + nicotinamide + pteroylglutamic acid	4·49 ± 0·22	29·23 ± 0·98	3·70 ± 0·24	1·84 ± 0·06	5·5 ± 0·17	4·7 ± 0·24
Basal + nicotinamide + choline + pteroylglutamic acid	4·00 ± 0·50	32·35 ± 1·31	3·59 ± 0·56	2·00 ± 0·11	6·1 ± 0·27	5·2 ± 0·20

Table 2. *Changes in weight of body organs of aminopterin-fed animals*

Animals on the laboratory stock diet (see text) were administered 225 μg. of aminopterin subcutaneously during 12 days, when they were killed. Nicotinamide was given orally at 25 mg./day/animal for this period. Results are the means from four animals in each group ± S.E. and are expressed as g./100 g. body wt.

Diet	Liver	Kidney	Spleen
Basal	4·02 ± 0·10	0·93 ± 0·01	0·30 ± 0·02
Basal + nicotinamide	4·57 ± 0·33	1·06 ± 0·04	0·36 ± 0·02
Basal + aminopterin	4·38 ± 0·12	1·11 ± 0·08	0·36 ± 0·03
Basal + aminopterin + nicotinamide	4·76 ± 0·14	1·18 ± 0·04	0·38 ± 0·03

Table 3. *Urinary excretion of nicotinic acid,* N^1*-methylnicotinamide and creatine*

Animals were grouped at end of 5 weeks on a pteroylglutamic acid-free purified ration (see text). Nicotinamide and choline supplementation were at 0·5% levels each, mixed in the diet. Pteroylglutamic acid (100 μg./animal/day) was administered intraperitoneally. Urine collections were for 3 days, 3 weeks after grouping. Results are averages for the pooled samples from four animals in each group ± S.E.M. and are expressed as mg./100 g. body wt./day.

Diet	Leucocytes/mm.^{3} (× 1000)	Total nicotinic acid	N^1-methylnicotinamide	Total creatine
Basal (no pteroylglutamic acid)	7·82 ± 0·45	0·31 ± 0·13	0·12 ± 0·03	1·1 ± 0·08
Basal + nicotinamide	6·56 ± 0·60	2·81 ± 0·20	0·48 ± 0·04	1·0 ± 0·04
Basal + nicotinamide + choline	7·81 ± 0·36	2·12 ± 0·23	0·73 ± 0·03	1·3 ± 0·14
Basal + pteroylglutamic acid	10·69 ± 0·78	0·25 ± 0·10	0·22 ± 0·02	1·4 ± 0·05
Basal + nicotinamide + pteroylglutamic acid	10·31 ± 0·49	1·68 ± 0·14	0·80 ± 0·04	1·2 ± 0·08
Basal + nicotinamide + choline + pteroylglutamic acid	10·62 ± 0·31	1·29 ± 0·25	0·83 ± 0·03	1·6 ± 0·04

That nicotinamide toxicity is caused by a depletion of labile methyl groups is thus inferable from the increased urinary excretion of N^1-methylnicotinamide and decreased liver stores of choline and methionine. These changes also manifest themselves in increased liver lipids and decreased phospholipids. It is further seen that the nicotinamide effect is greater in animals rendered deficient in pteroylglutamic acid either by dietary means or by aminopterin feeding. Choline alleviates this toxicity. These results generally bear out recent reports on the mediation of pteroylglutamic acid and vitamin B_{12} in methyl metabolism (Arnstein & Neuberger, 1953; Stekol, Weiss, Smith & Weiss, 1953).

Choline oxidase. There is decreased choline oxidation in pteroylglutamic acid deficiency either by dietary means (Table 4) or by aminopterin (Table 5). A lowering in choline oxidase activity is also observable as a result of nicotinamide feeding in both the pteroylglutamic acid deficient and normal groups. Handler & Bernheim (1942) have observed a reduced choline oxidase activity in fatty livers. The activity of the oxidase is also known to be influenced by pteroylglutamic acid (Williams, Sreenivasan, Sung & Elvehjem, 1953*b*) and vitamin B_{12} (Williams *et al.* 1953*a*). One of the reasons for the lowered choline oxidase activity in aminopterin-fed rats is stated to be a decreased synthesis of ascorbic acid and consequent impairment in the conversion of pteroylglutamic acid into folinic acid (Williams, 1951).

Xanthine oxidase. Several reports point to a relationship of pteroylglutamic acid to xanthine oxidation (Fatterpaker & Sreenivasan, 1951). The lability of the enzyme is also shown from observations on variations in its activity as a result of dietary changes (Litwack, Williams, Feigelson & Elvehjem, 1950; Dhungat & Sreenivasan, 1952). Since methionine is apparently essential in controlling xanthine oxidase activity, it was of interest to follow changes in this enzyme in the present work. Results (Tables 4 and 5) show that nicotinamide feeding reduces xanthine oxidase activity, presumably in part as a result of methyl depletion, and that *in vitro* synthesis of the enzyme is generally in proportion to the decrease in the liver xanthine oxidase activity (cf. Dhungat & Sreenivasan, 1952).

Table 4. *Changes in liver enzymes, pantothenic acid and adrenaline*

For explanation of table, see Tables 1 and 3 and text. Results are averages for four animals in each group ± S.E.M.

Diet	Choline oxidase (μl. O_2/g. dry wt./hr.)	Xanthine oxidase activity (μl. O_2/g. dry wt./2 hr.)	Xanthine oxidase synthesis *in vitro* (μl. O_2/g. dry wt./2 hr.)	Pantothenic acid (μg./g. dry liver)	Coenzyme A (μg./sulphanilamide acetylated/g. dry liver)	Adrenaline* (μg./100 mg. fresh adrenal glands)
Basal (no pteroylglutamic acid)	1530±104	252±18	348±17	285±18	260±13	83±4
Basal + nicotinamide	1250±112	192±17	307±26	176±12	202±11	71±3
Basal + nicotinamide + choline	1695±126	258±24	195±15	264±21	360±24	97±5
Basal + pteroylglutamic acid	2120±130	249±22	272±28	247±7	291±19	111±6
Basal + nicotinamide + pteroylglutamic acid	1835±95	195±25	215±36	180±10	205±8	117±4
Basal + nicotinamide + choline + pteroylglutamic acid	2170±142	293±16	202±0	285±26	362±29	115±1

* Pooled adrenals from two rats were used for assay in duplicate.

Table 5. *Nicotinamide toxicity in aminopterin-fed rats*

For explanation, see Table 2. All determinations were carried out in duplicate on four independent samples in each group. Results are mean values ± S.E.

Diet	Leucocytes/mm.^{3} (×1000)	Total lipids (g./100 g. fresh liver)	Phospholipids (g./100 g. fresh liver)	Choline oxidase (μl. O_2/g. dry wt. in 2 hr.)	Liver xanthine oxidase (μl. O_2/g. dry wt./2 hr.)	Xanthine oxidase synthesis *in vitro* (μl. O_2/g. dry wt./2 hr.)	Adrenaline* (μg./100 mg. fresh adrenals)
Basal	10·78±0·15	4·01±0·06	1·78±0·09	2820±70	744±66	716±52	98±6
Basal + nicotinamide	10·48±0·12	4·15±0·10	1·34±0·03	2440±115	466±89	640±32	89±1
Basal + aminopterin	7·66±0·16	4·95±0·03	1·36±0·07	2280±65	288±76	452±41	72±4
Basal + aminopterin + nicotinamide	7·81±0·31	5·55±0·24	1·14±0·04	1880±222	131±69	318±50	62±3

* See footnote to Table 4.

Pantothenic acid and coenzyme A. These determinations were made in view of various observations correlating pantothenic acid and coenzyme A with pteroylglutamic acid and vitamin B_{12}. Yacowitz, Norris & Heuser (1951) had reported a sparing action by vitamin B_{12} for pantothenic acid in chicks. That vitamin B_{12} may also aid in the transfer of pantothenic acid from livers of chicks was suggested by Evans, Groschke & Butts (1951). More recently, it has been observed by Boxer, Ott & Shonk (1953) that there is a fivefold increase in coenzyme A concentration in livers of vitamin B_{12}-deficient chicks. A sparing action of methionine for pantothenic acid in antibody formation was noted by Ludovici, Axelrod & Carter (1951). According to Popp & Totter (1952), pteroylglutamic acid deficiency resulted in decreased liver stores of coenzyme A. An involvement of pantothenic acid in the control of adrenal function is recognized (Hurley & Morgan, 1952).

The pantothenic acid estimations in the present studies were carried out by a method (Skeggs & Wright, 1944) which has since been shown to give low results (Novelli, Kaplan & Lipmann, 1949) owing to incomplete liberation of bound forms. Nevertheless, a general lowering of pantothenic acid content is seen in nicotinamide toxicity (Table 4). This is also the case with coenzyme A. However, simple pteroylglutamic acid deficiency does not show itself in any significant changes in liver levels of pantothenic acid or coenzyme A. The effect of choline on coenzyme A stores is marked.

Adrenaline. An enlargement of the adrenals in the aminopterin-fed animals as well as due to nicotinamide toxicity was referred to earlier. This was followed by determination of the changes in adrenaline content of the adrenals in the different groups.

Keller, Boissonnas & du Vigneaud (1950) showed that phenylalanine and methionine are the precursors for adrenaline and that a transmethylation is involved. It is known that methionine deficiency can lower adrenaline synthesis in rats (Hutcheon & Parker, 1950). The present studies (Tables 4 and 5) show the relationship of dietary pteroylglutamic acid to adrenaline as well as the effect of nicotinamide toxicity on this hormone in pteroylglutamic acid deficiency. These results are consistent with earlier observations that pteroylglutamic acid is involved in other methylation reactions, such as creatine and N^1-methylnicotinamide formation (Fatterpaker *et al.* 1951, 1952*a*). In a recent report, D'Iorio & Plaut (1952) observed that in hyperthyroid rats the adrenaline content is decreased and that vitamin B_{12} partially restores this condition. The potentiating effect of vitamin B_{12} on pteroylglutamic acid in transmethylating systems has been discussed by Sreenivasan (1951).

Adrenaline secretion is known to be influenced by a number of experimentally uncontrollable stress and emotional factors. Hence the influence of pteroylglutamic acid on adrenaline metabolism has been confirmed indirectly in other studies on the reversible glycogen to glucose transformation in isolated rat diaphragms from normal and aminopterin-fed rats (to be published).

The involvement of pteroylglutamic acid in adrenaline biosynthesis is apparently indirect as with the creatine- and the N^1-methylnicotinamide-synthesizing system (Fatterpaker *et al.* 1952*b*, 1954).

SUMMARY

1. Incorporation of 0·5% nicotinamide in a pteroylglutamic acid-free purified ration for young albino rats resulted in forced methylation and consequent depletion of liver choline and methionine as well as to increase in neutral lipids and to decrease in phospholipids.

2. Pteroylglutamic acid deficiency or nicotinamide feeding also resulted in a decrease in liver choline oxidase activity; a similar but somewhat less pronounced effect on liver xanthine oxidase activity was also reflected in correspondingly increased ability of the liver slices for synthesis of the enzyme *in vitro*.

3. Adrenaline content of the adrenals is significantly decreased in nicotinamide-fed pteroylglutamic acid-deficient animals.

4. The foregoing effects of nicotinamide toxicity are corrected by pteroylglutamic acid (100 μg./day/rat) and/or 0·5% dietary choline.

5. There is no marked change in liver stores of pantothenic acid or of coenzyme A as a result of pteroylglutamic acid deficiency. However, ingestion of nicotinamide results in somewhat lowered values for both forms of the vitamin in the liver. Choline supplementation increases liver levels of coenzyme A.

6. The alleviation by pteroylglutamic acid of nicotinamide toxicity has also been shown in studies with aminopterin-fed rats.

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Studies in the Biochemistry of Micro-Organisms

94. THE COLOURING MATTERS OF SPECIES IN THE *ASPERGILLUS NIDULANS* GROUP PART I. ASPERTHECIN, A CRYSTALLINE COLOURING MATTER OF *ASPERGILLUS QUADRILINEATUS* THOM & RAPER

By B. H. HOWARD and H. RAISTRICK
Department of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

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One of the outstanding characteristics of the *Aspergillus nidulans* group of species of moulds is that they usually produce ascospores contained in dark-coloured perithecia. The ascosporic members of the group consist of six recognized species or varieties (Thom & Raper, 1945) in cultures of which the colony reverse, the perithecial walls and the ascospores are usually all reddish purple to dark purple-red in colour. The ascospores are characterized by equatorial bands which are generally two in number, but number four in one member of the group, *A. quadrilineatus* Thom & Raper—hence its specific epithet. We have investigated the colouring matters present in two highly pigmented strains of this species which were isolated in this Department from different samples of Australian soil, and have isolated from each of them in pure crystalline form a hitherto undescribed anthraquinone colouring matter for which we propose the name asperthecin. However, since the crude colouring matters isolated

from the two strains, S.M. 200 and S.M. 297, showed considerable chemical differences they will be described separately.

A. quadrilineatus strain S.M. 200 was cultivated on Raulin–Thom solution. Microscopic examination of these mature cultures, mounted in dilute aqueous sodium bicarbonate solution, showed that the dark purple-red colour of the perithecia and ascospores diffused and gave a purple-blue solution. No such effect was observed in cultures mounted in water. Further, when portions of mature mycelium, pressed as dry as possible on filter paper, were immersed in cold concentrated sulphuric acid a very intense purple colour was immediately produced.

The isolation, in a pure crystalline form, of the colouring matter, asperthecin, responsible for these reactions, was found to be unusually difficult and proved to be impossible by solvent extraction of the vacuum-dried mycelium. It was finally accomplished by disintegrating the freshly harvested and washed mycelium in aqueous N hydrochloric acid in a high-speed macerator. The resulting suspension was filtered, washed with water and drained thoroughly on the pump. The brown friable mass, while still moist, was extracted with large volumes of cold absolute ethanol. The orange-brown extract was evaporated to low bulk *in vacuo* and gave crude asperthecin in orange-brown needles. The average yield obtained corresponded to about 15 g./100 flasks containing a total of 35 l. of Raulin–Thom solution and is calculated to be, on the average, about 2·5 % of the dry weight of the mycelium.

Purification of the crude crystalline asperthecin by direct crystallization proved to be unsatisfactory. It was, however, readily purified via the crystalline hexaacetate, which was then hydrolysed to the parent colouring matter by boiling in methanol containing a little sulphuric acid. The recovered asperthecin then crystallized readily either from methanol as chestnut-brown needles or from dioxan as bright red needles containing solvent of crystallization.

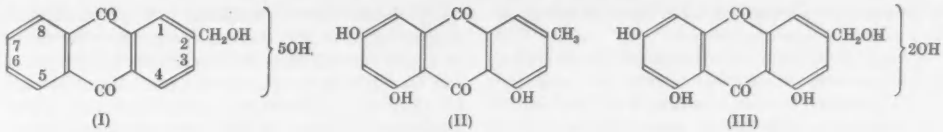
Asperthecin, $C_{15}H_{10}O_8$, darkens but does not melt when heated to 370°. It contains no methoxyl group, or methyl group attached to carbon. It is almost insoluble in water and is not readily soluble in any organic solvent except pyridine. Its reddish orange solutions in organic solvents, including glacial acetic acid, do not fluoresce in daylight nor does its stable, intensely purple solution in cold concentrated sulphuric acid. It gives a stable purple ferric colour in ethanolic solution. It is readily soluble in dilute sodium bicarbonate, sodium carbonate and sodium hydroxide, giving purple-red to purple solutions which are increasingly unstable in air with rise in pH, so that the initially purple solution in aqueous sodium hydroxide fades in a few seconds to orange and becomes colourless overnight.

The following crystalline functional derivatives of asperthecin were prepared and characterized. (*a*) Hexaacetylasperthecin, $C_{15}H_4O_2(O.CO.CH_3)_6$, by heating with acetic anhydride and a little concentrated sulphuric acid; pale yellow needles, m.p. 228°, optically inactive and insoluble in aqueous sodium carbonate. (*b*) Pentamethylasperthecin, $C_{15}H_5O_3(OCH_3)_5$, by prolonged methylation with dimethyl sulphate and anhydrous potassium carbonate in acetone; orange plates, m.p. 134–135°, which are insoluble in alkalis, even aqueous sodium hydroxide. (*c*) Dimethylasperthecin, $C_{15}H_8O_6(OCH_3)_2$, by brief methylation with diazomethane in acetone–ether; bright red blades, m.p. 236–237°, which are readily soluble in aqueous sodium carbonate. (*d*) Dimethylasperthecin tetraacetate, $C_{15}H_4O_2.(OCH_3)_2.(O.CO.CH_3)_4$, by acetylation of (*c*) with acetic anhydride and concentrated sulphuric acid; yellow rods, m.p. 229–230°.

Consideration of the properties of asperthecin already described shows that the pigment displays the character of a polyhydroxyquinone. Further, since it has also been shown that, on distillation with electrolytic zinc dust, asperthecin gives 2-methylanthracene, it follows that it must be a hexahydroxyanthraquinone since, as reported above, it gives, on acetylation, a hexaacetyl derivative, $C_{15}H_4O_2(O.CO.CH_3)_6$. Hence the functions of all the eight oxygen atoms are accounted for. So far as we are aware no natural polyhydroxyanthraquinone of biological origin, other than asperthecin, contains six hydroxyl groups.

The following evidence indicates that the side chain present in asperthecin which gives rise to a methyl group on zinc dust distillation is a methylol (CH_2OH) group. (i) Asperthecin does not contain a carbon-methyl (C-CH_3) grouping as was shown by a Kuhn–Roth estimation. (ii) Asperthecin does not contain a carboxyl (COOH) group, since its hexaacetate is not soluble in aqueous sodium carbonate. (iii) Asperthecin only gives a penta- and not a hexa-methyl ether on prolonged methylation with dimethyl sulphate and anhydrous potassium carbonate in acetone, and this pentamethyl ether is not soluble in aqueous sodium hydroxide.

The partial structure of asperthecin may therefore be represented as structure (I), and it now remains to determine the orientation of the five nuclear hydroxyl groups. The first evidence on this point is provided by the finding that when asperthecin is heated with concentrated hydriodic acid and red phosphorus in glacial acetic acid solution, a reduction product (almost certainly emodin anthranol) is formed which gives emodin (4:5:7-trihydroxy-2-methylanthraquinone, II) on oxidation with chromic acid in acetic acid solution.

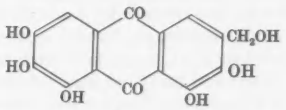


(I) (II) (III)

Emodin

Hence, assuming that no migration of hydroxyl groups occurs during the reduction, the partial structure of asperthecin may be expanded to (III).

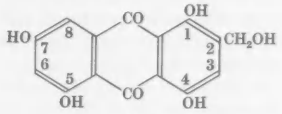
The evidence available at present for the orientation of the two remaining hydroxyl groups is as follows. In spite of repeated attempts, carried out under a variety of experimental conditions, we have completely failed to detect any fluorescence in daylight of any solutions of asperthecin, including its reddish orange solution in glacial acetic acid and its intense purple solution in cold concentrated sulphuric acid. Raistrick, Robinson & Todd (1934, p. 567) compared the colours and fluorescences of solutions in glacial acetic acid of eight synthetic and seven natural hydroxyanthraquinones and concluded that fluorescence is characteristic of quinizarin (1:4-dihydroxyanthraquinone) derivatives. In the intervening years a number of other polyhydroxyanthraquinones of established structure have been examined and have confirmed the validity of this generalization. The only way in which two nuclear hydroxyl groups can be introduced into structure (III) which will avoid formulating asperthecin as a derivative of quinizarin is structure (IV). Hence, unless asperthecin is an exception to the above generalization, the two remaining hydroxyl groups must be in positions 3 and 6. This conclusion is supported by the fact that, although solutions of asperthecin did show a weak fluorescence when viewed in ultraviolet light, this fluorescence was similar in colour to, but slightly less intense than, that given by emodin (II) in the same solvents.



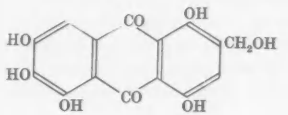
(IV)

On this evidence asperthecin is 3:4:5:6:7-pentahydroxy-2-hydroxymethylanthraquinone (structure IV) and as will be shown later this structure illustrates certain properties of asperthecin which are unique among polyhydroxyanthraquinones of mycological origin and rare among those from any natural source.

Structure (IV), however, does not clearly illustrate the ultraviolet and infrared absorption spectra of asperthecin. Birkinshaw (see Appendix to this paper) has determined the ultraviolet spectra of a number of natural and synthetic polyhydroxyanthraquinones of established structure and has shown that, of nine polyhydroxyanthraquinones examined, the ultraviolet spectrum of catenarin, 1:4:5:7-tetrahydroxy-2-methylanthraquinone (Anslow & Raistrick, 1940, 1941) is most closely related to that of asperthecin. Since the ultraviolet spectrum of the unknown ω-hydroxycatenarin (V) would be expected to be practically the same as that of catenarin in the λ200–600 mμ. region, as is known to be the case with emodin and ω-hydroxyemodin, we may deduce from this evidence that asperthecin is a hydroxy derivative of ω-hydroxycatenarin of which three are possible, i.e. (*a*) 3-hydroxy-, (*b*) 6-hydroxy-, and (*c*) 8-hydroxy-ω-hydroxycatenarin.



(V)



(VI)

Of the three possibilities (*c*), the 8-hydroxy compound, is the least probable structure for asperthecin since it contains only one β-hydroxyl group and hence, unlike asperthecin, would probably be insoluble in aqueous sodium bicarbonate. Further, (*c*) would be a simple derivative of the known 8-hydroxycatenarin, 1:4:5:7:8-pentahydroxy-2-methylanthraquinone, which dissolves in concentrated sulphuric acid to a pure blue solution with a red fluorescence (Raistrick *et al.* 1934, p. 570), whereas asperthecin gives a purple non-fluorescent solution in the same reagent. It is also improbable that asperthecin is the 3-hydroxy derivative (*a*), since the closely related 1:3:5:7-tetrahydroxy-2:6-dimethylanthraquinone is, unlike asperthecin, insoluble in aqueous sodium bicarbonate (Kusaka, 1935, quoted by Asahina & Shibata, 1954, p. 156). The 6-hydroxy derivative (*b*) (structure VI) therefore seems to be the most probable of the three possible hydroxy derivatives, (*a*), (*b*) and (*c*), of ω-hydroxycatenarin. On this

evidence asperthecin is 1:4:5:6:7-pentahydroxy-2-hydroxymethylanthraquinone.

Briggs, Nicholls & Paterson (1952) found that in the ultraviolet absorption spectra of hydroxyanthraquinones the major absorption band due to the carbonyl group of the quinonoid nucleus is greatly affected by the number of free, i.e. unsubstituted, α-hydroxyl groups, the presence of β-hydroxyl groups having little effect. Thus, in hydroxyanthraquinones containing no free α-hydroxyl groups this band occurs at 356–362·5 mμ., with one free α-hydroxyl group at 407·5–413·5 mμ. and with two such groups at 427·5–432·5 mμ. These figures are broadly confirmed by Birkinshaw (see Appendix to this paper) and amplified by the observation that in three anthraquinones, each having three free α-hydroxyl groups, the above-mentioned bands give place to new bands in the 490 and 510 mμ. regions, i.e. islandicin (1:4:5-trihydroxy-2-methylanthraquinone) at 492 and 513 mμ.; helminthosporin (4:5:8-trihydroxy-2-methylanthraquinone) at 480, 490 and 510 mμ.; and catenarin (1:4:5:7-tetrahydroxy-2-methylanthraquinone) at 488·5 and 508 mμ. It is therefore perhaps significant that asperthecin has bands at 484 and 510 mμ. and that structure (VI) has three free α-hydroxyl groups while structure (IV) has only two. On this evidence structure (VI) is to be preferred to structure (IV). On the other hand, since any anthraquinone having three free α-hydroxyl groups must of necessity be a derivative of quinizarin (1:4-dihydroxyanthraquinone) it is curious that while islandicin, helminthosporin and catenarin all give markedly fluorescent solutions in glacial acetic acid, asperthecin shows no such fluorescence.

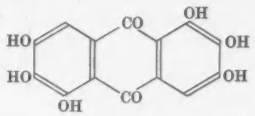
The infrared spectrum of solid asperthecin has also been examined. Flett (1948) examined two mono-, five di-, one tri-, and one tetra-hydroxyanthraquinone in the solid state and concluded that a band in the region of 1670–1680 cm.$^{-1}$ in the infrared absorption spectrum is characteristic of a carbonyl group which is not chelated with an α-hydroxyl group, while a band in the 1630–1640 cm.$^{-1}$ region indicates a carbonyl group chelated with an α-hydroxyl group. The infrared spectrum of solid asperthecin has a broad absorption band in the double-bond stretching region with a maximum at 1606 cm.$^{-1}$ and a shoulder at about 1575 cm.$^{-1}$. No band was, however, observed near 1670 cm.$^{-1}$. Hence, if one accepts Flett's correlation rule it follows that both the C=O groups in asperthecin must be chelated with α-hydroxyl groups, so that structure (IV) for asperthecin, which contains only one chelated carbonyl group, would be untenable. However, workers at the Butterwick Research Laboratories of Imperial Chemical Industries Ltd. (L. A. Duncanson & J. F. Grove, private communication) have examined 19 polyhydroxyanthraquinones and derivatives in the solid state and have shown that the correlation rules worked out by Flett on simple compounds cannot always be applied to polyhydroxyanthraquinones. Thus, although alizarin (1:2-dihydroxyanthraquinone) shows a strong absorption band at 1664 cm.$^{-1}$, anthragallol (1:2:3-trihydroxyanthraquinone) shows only a weak shoulder at about 1660 cm.$^{-1}$. Hence, since structure (IV) for asperthecin contains the 1:2:3-trihydroxyanthraquinone structure, it appears certain that structure (IV) cannot be eliminated on the infrared evidence alone.

However, analysis of the Butterwick Laboratory infrared spectra seems to indicate a correlation between the frequency of the strongest absorption band in the 6 μ. (1670 cm.$^{-1}$) region and the number and position of free α-hydroxyl groups in polyhydroxyanthraquinones produced by moulds and containing two or more α-hydroxyl groups. Thus, in cynodontin with four α-hydroxyl groups (1:4:5:8-tetrahydroxy-2-methylanthraquinone) the band is at 1580 cm.$^{-1}$; in four substances with three α-hydroxyl groups the band is close to 1600 cm.$^{-1}$, i.e. helminthosporin (4:5:8-trihydroxy-2-methyl-) at 1602, islandicin (1:4:5-trihydroxy-2-methyl-) at 1598, catenarin (1:4:5:7-tetrahydroxy-2-methyl-) at 1600, and erythroglaucin (1:4:5-trihydroxy-7-methoxy-2-methyl-) at 1600; and in four substances with two hydroxyl groups in the α-position to the same carbonyl group the band is close to 1625 cm.$^{-1}$, i.e. emodin (4:5:7-trihydroxy-2-methyl-) at 1623, ω-hydroxyemodin (4:5:7-trihydroxy-2-hydroxymethyl-) at 1625, physcion (4:5-dihydroxy-7-methoxy-2-methyl-) at 1627, and chrysophanol (4:5-dihydroxy-2-methyl-) at 1627. Since the corresponding band in asperthecin is at 1606, it seems logical to conclude that asperthecin contains, in addition to other substituents, the 1:4:5-trihydroxy grouping, a conclusion which is in agreement with the ultraviolet absorption spectra findings and with structure (VI) for asperthecin.

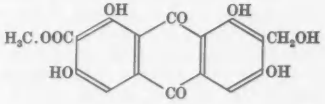
It is thus clear that, while both structures (IV) and (VI) illustrate many of the properties and reactions of asperthecin, neither structure explains all of them. It now remains to examine which of these structures best illustrates the other unusual properties of asperthecin previously referred to, which are:

(*a*) Asperthecin has the unusual property that it is readily soluble in aqueous sodium bicarbonate although it does not contain a —COOH group, as is indicated by its empirical formula and by the fact that its hexaacetate is not soluble in sodium carbonate. It is a generally accepted rule that polyhydroxyanthraquinones having only α-hydroxyl groups, i.e. in positions 1, 4, 5 or 8, dissolve in aqueous sodium hydroxide but do not dissolve in

aqueous sodium carbonate or in sodium bicarbonate, while those having β-hydroxyl groups, i.e. in positions 2, 3, 6 or 7, whether with or without α-hydroxyl groups, dissolve in aqueous sodium hydroxide or carbonate but rarely in sodium bicarbonate. It appears probable that this generalization must be modified in the case of those polyhydroxyanthraquinones which have two or more β-hydroxyl groups. Thus, while anthragallol (1:2:3-trihydroxyanthraquinone) with two β-hydroxyl groups is insoluble in sodium bicarbonate, the following are all soluble in this reagent: rufigallic acid (VII), synthesized by Robiquet (1836) from gallic acid, which has four β-hydroxyl groups; asperthecin (if IV) which has three β-hydroxyl groups; asperthecin (if VI) and 1:3:6:8-tetrahydroxyanthraquinone (Shibata, 1941) and rhodocladonic acid (VIII), from a number of lichen species in the genus *Cladonia* (Zopf, 1907; Koller & Hamburg, 1936; Shibata, 1941), which have two β-hydroxyl groups. Rufigallic acid and asperthecin decompose very rapidly in strongly alkaline solution. This remarkable instability towards alkalis is generally believed to be due to the presence of three vicinal hydroxyl groups, a belief which is illustrated in both structures (IV) and (VI) for asperthecin.



(VII)
Rufigallic acid



(VIII)
Rhodocladonic acid

(*b*) Asperthecin is a powerful mordant dyestuff and dyes white wool, mordanted with chromium, aluminium or tin, an intense indigo blue, deep violet or dull red colour respectively. This property which, among polyhydroxyanthraquinones, is characteristic of derivatives of alizarin (1:2-dihydroxyanthraquinone), is in conformity with structure (IV) for asperthecin which contains two 1:2-dihydroxy groupings and is therefore doubly an alizarin derivative, and also with structure (VI).

Chemical work on the constitution of asperthecin is proceeding. In the meantime we believe that asperthecin is either 3:4:5:6:7- or 1:4:5:6:7-pentahydroxy-2-hydroxymethylanthraquinone of structures (IV) or (VI) respectively. We have at present no evidence to decide conclusively between these structures.

The second strain of *A. quadrilineatus*, strain S.M. 297, was cultivated on Czapek–Dox or Raulin–Thom solutions and gave mycelia from each culture medium which, when freshly harvested, gave an intense prussian blue colour in cold concentrated sulphuric acid which was quite different from the deep purple colour given by mycelia of strain S.M. 200 in the same reagent. Isolation of the colouring matters of strain S.M. 297 by extraction of the pressed, disintegrated mycelium with ethanol, exactly as was done with strain S.M. 200, resulted in a light brown, mainly crystalline solid in a yield of about 5 g./100 flasks which gave the typical intense prussian blue sulphuric acid colour observed with the intact mycelium. However, all attempts to purify this crude material and to isolate from it the pure colouring matter giving the blue sulphuric acid colour have so far failed and have led instead to the isolation of asperthecin in all cases. The methods used for purification included direct crystallization from dioxan; acetylation, which furnished an acetate having the same melting point and mixed melting point as hexaacetylasperthecin and giving pure asperthecin on hydrolysis; chromatography on calcium carbonate, which did indeed give two different coloured bands, which were separated mechanically, but yielded asperthecin in each case. Further, when the fresh ethanolic extract of the mycelium of strain S.M. 297 was diluted with ether and kept in the laboratory for a week, the initially reddish solution had changed colour to orange and the sulphuric acid colour of the colouring matter contained in it had changed from deep blue to purple.

It therefore seems very probable that the colouring matter giving the blue sulphuric acid colour is very unstable and is readily converted into asperthecin, possibly by tautomeric change to which both structures (IV) and (VI) would appear to lend themselves readily.

A preliminary survey has been carried out on a number of authentic strains of *Aspergillus nidulans* (Eidam) Wint. and other species in the *A. nidulans* group, many of which have long been in laboratory culture and were not highly pigmented. None of them proved to be nearly as rich in asperthecin as *A. quadrilineatus* strain S.M. 200. However, small amounts of crystalline material having the properties of asperthecin (colour; crystal form; solubility in sodium bicarbonate to a purple-red solution; deep purple solution in cold concentrated sulphuric acid) were obtained from cultures on Czapek–Dox solution of one strain each of *A. nidulans*, *A. nidulans* mut. *alba* Yuill and strain N.R.R.L. 521 of *A. quadrilineatus* from Dr K. B. Raper. Small amounts of amorphous material giving the colour

reactions of asperthecin were isolated from two other strains of *A. nidulans* and one strain of *A. rugulosus* Thom & Raper. There is thus good presumptive evidence for the belief that asperthecin will prove to be of frequent occurrence in species in the *A. nidulans* group, particularly in cultures recently isolated from natural sources.

EXPERIMENTAL

Cultures

Two strains of *Aspergillus quadrilineatus* Thom & Raper, both isolated here by Mrs S. Marcus, were used in this work. They were (*a*) Strain L.S.H.T.M. catalogue no. S.M. 200. Isolated in October 1950 from Australian soil sample 257·1. This light clay Stony Desert surface soil (0–1 in.), pH 6·8, was collected about 15 miles west of Tibooburra, New South Wales. This strain, which was identified by Mr G. Smith to whom we are indebted for the following notes, is predominantly perithecial on all culture media. Cultures show little green colour, being mostly thick grey layers of hülle cells in which the purple perithecia are embedded. The ascospores are quite typical, the four equatorial bands (the origin of the specific epithet) being quite as evident as in the type strain N.R.R.L. 201 received from Dr Kenneth B. Raper. (*b*) Strain L.S.H.T.M. catalogue no. S.M. 297. Isolated in February 1951 from Australian soil sample 221·1. This very loose red sandy surface soil (0–½ in.), pH 7·4, was collected from the sand ridge country on the New South Wales–South Australian border. The culture was identified by Dr K. B. Raper as a somewhat abnormal strain of *A. quadrilineatus*. It is quite normal in colony appearance, but is somewhat abnormal in the unusual indistinctness of the two outer of the four ridges on the ascospores, demonstration of which with certainty demands the limit of resolution of the microscope.

It is of interest to note that both soils were from the most arid situations of the very low rainfall area of New South Wales.

We wish to thank Professor E. G. Hallsworth, now Head of the Department of Agricultural Sciences of the University of Nottingham, for the gift of a number of Australian soil samples including the two samples referred to above.

THE COLOURING MATTER OF *A. QUADRILINEATUS* STRAIN S.M. 200

We have found, by microscopic examination of cultures of this strain of various ages and mounted in a variety of media, that the characteristic dark red or purple colour of the perithecial walls and ascospores is due to the presence, probably in the form of a salt, of a hitherto undescribed colouring matter for which we propose the name *asperthecin*. Thus, immature cultures, containing only hyphae and hülle cells, showed no colour when mounted in any medium. Mature perithecia however appeared dark blue to black in aqueous suspension and orange in lacto-phenol. In dilute aqueous $NaHCO_3$ the colour diffused rapidly from the perithecia and more slowly from the ascospores, giving a purple-blue solution.

Cultural conditions and characteristics

Raulin–Thom solution (glucose, 75·0 g.; tartaric acid, 4·0 g.; ammonium tartrate, 4·0 g.; $(NH_4)_2HPO_4$, 0·6 g.; $(NH_4)_2SO_4$, 0·25 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $FeSO_4$, $7H_2O$, 0·07 g.; $ZnSO_4$, $7H_2O$, 0·07 g.; distilled water, 1500 ml.) was distributed in 350 ml. amounts in 1 l. conical flasks. The flasks were plugged with cotton wool, sterilized and inoculated with a suspension in water prepared from mature cultures on slopes of Czapek–Dox or beer wort agar of *A. quadrilineatus* strain S.M. 200. The inoculated flasks were incubated for varying periods at 24° in the dark. During growth the surface of the mycelium became deeply folded, granular in texture and a dark buff colour, with a dark brown reverse. The culture fluid became a dull yellow colour. A number of cultural details including yields of asperthecin are collected in Table 1, in which, for strain S.M. 200, each batch contained 25 flasks. The weight of mycelium if dried is estimated to be about 150 g./25 flasks.

At intervals during the incubation period portions of the mycelium were pressed between filter papers and immersed in cold conc. H_2SO_4 or aqueous N-Na_2CO_3 or aqueous N-NaOH. In H_2SO_4 the mycelium gave after 7 days a moderate purple, after 14 days a strong violet and after 21 days a very dark purple colour. In Na_2CO_3 and NaOH the mycelium gave little or no colour after 7 or 14 days, but after 21 days gave a moderate purple in both reagents. Single flasks, examined after 21 days' growth, yielded approximately 6 g. of washed and dried mycelium.

Table 1. *Cultural details and yields of asperthecin or crude colouring matter from* Aspergillus quadrilineatus *strains*

Batch no.	Incubation period (days)	Residual glucose by polarimeter (%)	pH	Wt. of crude crystalline asperthecin (g.)
		Strain S.M. 200		
1	21	0·75	5·4	3·49
2	26	0·4	6·0	3·49
3	24	0·9	5·9	3·14
4	28	0·7	6·1	4·17
5	32	0·6	6·3	3·63
6	36	0·4	6·2	4·92
		Strain S.M. 297		
1	28	0·7	8·0	1·44*
2	38	0·5	8·1	1·97*

* Crude colouring matter.

Isolation of asperthecin

Considerable difficulty was experienced in attempting to isolate the colouring matter from the mycelium. Yields satisfactory in quantity and quality could be obtained only by careful attention to the following points. (i) Drying of the mycelium, even *in vacuo* and at temperatures below 45°, led to poor yields of impure material on subsequent solvent extraction. (ii) The use of hot solvents yielded material similar to that obtained in (i). (iii) The pigment could not be recovered satisfactorily from $CaCO_3$ chromatograms, being accompanied by a dark tarry impurity or decomposition product. (iv) Asperthecin is present in the mycelium, at least in part, as a salt which is insoluble in water and in organic solvents. Hence the mycelium must be treated with acid before the bulk of the colouring matter can be removed. (v) Asperthecin is contained very largely or perhaps wholly in the perithecia and ascospores. Hence mechanical disintegration of the perithecia before solvent extraction considerably facilitates removal of the colouring matter.

The best method of isolation of asperthecin was therefore found to be the following. At the end of the incubation period (for batch 6, 36 days at 24°) the culture fluid was separated from the mycelium by decanting through muslin and the mycelium was washed once with water. The mycelial mats were then suspended in aqueous N-HCl and disintegrated, five at a time, in a Top Drive Macerator (Townson and Mercer, Croydon). After 10 min. treatment, microscopic examination of the brown suspension showed the absence of intact cells apart from a few hülle cells and ascospores. The suspension was filtered through a hardened paper, washed with water until free from acid and drained thoroughly on the pump. The mycelium at this stage was a brown friable mass containing about 50% solids. It was roughly crumbled and vigorously stirred for 4 hr. with ethanol (2 l.). After standing overnight, the dark orange-brown ethanolic extract was separated, the solid re-stirred during 1 working day with ethanol (2·5 l.) and kept overnight. A total of six such extracts was made, the last being of a very pale brown colour. Each extract was evaporated *in vacuo*, the first to about 100 ml., the last to about 20 ml. In each case the solid which separated consisted mainly of orange-brown needles, accompanied in the first extract by a few colourless needles and rods and in the succeeding extracts by large quantities of 'fat'. The concentrated extracts were filtered separately, and the solids were washed first with a little ethanol and then copiously with light petroleum (b.p. 40–60°) until free from 'fat'. Yields of brown crystalline solid from batch 6 (1) 2·49 g.; (2) 1·08 g.; (3) 0·39 g.; (4) 0·34 g.; (5+6) 0·62 g.; total 4·92 g. from 25 flasks, corresponding to approximately 3·3% of the estimated wt. of dry mycelium. Only small amounts of very impure material could be obtained from the dark brown ethanol mother liquors, and 'fat' (21 g.) was left on evaporation of the light petroleum washings.

Purification and properties of asperthecin

Purification by direct crystallization of the crude crystalline product obtained above could not be effected with certainty and it was more convenient to convert it into the acetate, which was purified by crystallization and hydrolysed (see p. 482). The product of the hydrolysis of the pure acetate was a brownish red crystalline solid which could be crystallized from methanol or dioxan. Asperthecin forms chestnut-brown needles from methanol which darken but do not melt when heated to 370°. (Found, on material dried in high vacuum at 110° without loss in wt.: N, S, halogens, OCH_3, nil; C, 56·7; H, 3·2. $C_{15}H_{10}O_8$ requires C, 56·6; H, 3·2%.) Asperthecin separates from dioxan as bright red needles containing solvent of crystallization. (Found: loss on drying in high vacuum at 130°, 21·3%. $C_{15}H_{10}O_8.C_4H_8O_2$ requires 21·7%. Found on dried material: OCH_3, *C*-CH_3, nil; C, 56·6; 56·6, H, 3·2, 3·2. $C_{15}H_{10}O_8$ requires C, 56·6; H, 3·2%.)

Asperthecin is almost insoluble in water, light petroleum, $CHCl_3$ or benzene; slightly soluble in methanol or ethanol, and moderately soluble in acetone, glacial acetic acid and dioxan. Its solutions in organic solvents are reddish orange in colour and do not fluoresce in daylight. It dissolves very readily in pyridine, forming a pyridine salt which separates as purple needles on standing. Asperthecin dissolves in cold conc. H_2SO_4 to a stable purple non-fluorescent solution, which appears redder by transmitted light and bluer in thin layers, and in cold conc. HNO_3 to a blue solution which becomes purple, and then fades, on standing. It gives a stable purple ferric colour in ethanolic solution. It dissolves in boiling aqueous aluminium sulphate giving, after filtration, a clear purple, non-fluorescent solution which is almost indistinguishable in colour from an aqueous solution of potassium permanganate.

It is noteworthy that asperthecin is soluble in 0·5 N-$NaHCO_3$ to a stable purple-red solution. It dissolves in N-Na_2CO_3 to a purple-red solution, which is bluer than that in $NaHCO_3$ and fades overnight on standing in air with the formation of a blue precipitate; in aqueous N-NaOH it gives a purple solution which fades within a few seconds on exposure to air to a dull pale orange and becomes colourless overnight. Portions of a dilute ethereal solution of asperthecin were shaken with equal volumes of 0·2 M phosphate or borate buffers. At pH 4·0 and below the aqueous layer was colourless; at pH 5·0 it was very pale purple and at pH 6·0 a pale purple. At pH 7·2 the ether layer was pale orange and the aqueous layer a strong purple-red; with increasing pH the ether layer became still paler orange in colour until at pH 8·0 and above the ether layer was quite colourless and the aqueous layer a strong purple-red.

Addition of ethanolic potassium acetate (5%, w/v) to a hot ethanolic solution of asperthecin caused the immediate precipitation of the potassium salt as an amorphous, deep violet solid, insoluble in ethanol and organic solvents but readily soluble in water.

Asperthecin is chromatographically homogeneous, giving a uniform purple-pink band on $CaCO_3$ columns and on $MgCO_3$ a bluer more intensely coloured band, when chromatographed in ethanolic solution. It is reduced by zinc dust in glacial acetic acid solution, the colour changing quickly from orange to purple and then to pale yellow. $Na_2S_2O_4$ is without effect when added to a $NaHCO_3$ solution, but an excess of the reagent added to a solution in N-Na_2CO_3 changes the colour from purple-red to orange.

Derivatives of asperthecin

Hexaacetylasperthecin. Crude crystalline asperthecin (2·0 g.) was mixed with acetic anhydride (16 ml.) containing conc. H_2SO_4 (0·3 ml.). The mixture became warm, and yellow needles separated from the clear solution. After 10 min. the mixture was heated at 100° for 5 min. to complete the reaction. The brown solution was kept at 0° and the crystals were collected on a sintered glass filter, washed with

glacial acetic acid then with much water and dried. The slightly orange-yellow product (2·4 g.), m.p. 212–215°, was crystallized twice from glacial acetic acid (25 ml.) and a portion (0·10 g.) of the resulting material was purified for analysis by crystallizing several times from ethanol (100 ml.) giving *hexaacetylasperthecin* as pale yellow needles, m.p. 228°. (Found, on material dried in high vacuum at 110°: OCH_3, nil; C, 56·9, 56·85; H, 4·0, 3·8; CH_3CO, 45·2; mol.wt. (cryoscopic in camphor) 531, 529. $C_{27}H_{22}O_{14}$ requires C, 56·8; H, 3·9; $6CH_3CO$, 45·3%; mol.wt. 570.) Hexaacetylasperthecin shows no optical activity in $CHCl_3$ solution (*c*, 3·90), is insoluble in 0·5N-$NaHCO_3$ and N-Na_2CO_3 and gives no ferric colour in ethanol.

The original acetic anhydride filtrate and acetic acid washings were treated with ice water in the usual way to yield crude hexaacetylasperthecin as an orange amorphous solid (0·7 g.) which melted indefinitely from 185° upwards and was most conveniently purified by crystallizing from a small volume of the acetic anhydride–conc. H_2SO_4 mixture.

Hydrolysis of pure hexaacetylasperthecin A solution of pure hexaacetylasperthecin (0·78 g.) in methanol (70 ml.) containing 3% (v/v) of conc. H_2SO_4 was boiled under reflux for 1·5 hr. The solid dissolved to an orange solution during 5 min. and asperthecin began to separate as reddish needles after 30 min. The crystals were collected after storing overnight at 0° giving purified asperthecin as a brownish red solid (0·40 g.) which was crystallized from methanol or dioxan (see p. 481). The orange-red methanol filtrate was poured into hot water (350 ml.) from which the rest of the colouring matter (0·04 g.) separated on cooling.

Pentamethylasperthecin. A mixture of asperthecin (0·10 g.), dry acetone (80 ml.), dimethyl sulphate (1·0 ml.) and freshly ignited K_2CO_3 (2·0 g.) was boiled under reflux. The mixture soon went purple and then slowly became brown. After 14 hr. boiling, the blue solid was removed by filtration and the solvent was evaporated from the filtrate giving an oily orange residue which was shaken with aqueous 0·2N-NaOH to remove dimethyl sulphate. The residue was sublimed in high vacuum at 180°, giving an oily orange sublimate which was crystallized twice from ethanol–light petroleum (b.p. 60–80°). *Pentamethylasperthecin* was obtained as large flat orange plates, m.p. 134–135°. (Found, on material dried in high vacuum at room temp.: C, 61·4, 61·7; H, 5·4, 5·2; OCH_3, 39·6, 39·5. $C_{20}H_{20}O_8$ requires C, 61·85; H, 5·2; $5OCH_3$, 40·0; $C_{21}H_{22}O_8$ requires C, 62·7; H, 5·5; $6OCH_3$, 46·3%.) Pentamethylasperthecin is insoluble in alkalis, gives a stable purple-blue solution in cold conc. H_2SO_4 and a red solution in cold conc. HNO_3.

Dimethylasperthecin. Asperthecin (1·2 g.) was ground under acetone (30 ml.) to a fine suspension. An ethereal solution (400 ml.) of diazomethane (from 3 g. of nitrosomethylurea) was quickly added. A brick-red precipitate of needles separated, and after standing for 2 hr. at room temp. the mixture was concentrated to 50 ml. and kept overnight at 0°. The crystals were collected as a bright brick-red solid (1·07 g.) which was purified by crystallization from dioxan and then ethanol giving *dimethylasperthecin* as clusters of thin blades, bright red in bulk, m.p. 236–237°. (Found, on material dried in high vacuum at 120°: C, 58·8, 58·9; H, 4·2, 4·2; OCH_3, 18·0, 18·2. $C_{17}H_{14}O_8$ requires C, 58·9; H, 4·1; $2OCH_3$, 17·9%.) Dimethylasperthecin gives generally similar colour reactions to asperthecin itself, except that it is not soluble in 0·5N-$NaHCO_3$. In N-Na_2CO_3 it gives a red-violet solution, and in aqueous N-NaOH a violet solution, both solutions being slightly bluer and more stable than the corresponding asperthecin solutions; cold conc. H_2SO_4, stable purple-blue solution; cold conc. HNO_3, blue solution; ethanolic $FeCl_3$, stable purple solution, slightly browner than that given by asperthecin. Unlike asperthecin it is readily reduced by $Na_2S_2O_4$. The reduction product gives an orange-yellow solution in ether with a green fluorescence, and a yellow solution in N-Na_2CO_3.

Dimethylasperthecin tetraacetate. Dimethylasperthecin (0·9 g.) was heated for 15 min. at 100° with acetic anhydride (3 ml.) containing 2% (v/v) conc. H_2SO_4. Yellow crystals soon began to separate, and, after chilling, the semi-solid mass was transferred to a sintered glass filter and washed first with glacial acetic acid and then with much water. The dried product (1·17 g.) was crystallized from acetic anhydride (7 ml.) yielding *dimethylasperthecin tetraacetate* as yellow pointed rods, m.p. 229–230°. (Found, on material dried in high vacuum at 120°: C, 58·3; H, 4·5; OCH_3, 12·3; CH_3CO, 32·2. $C_{25}H_{22}O_{12}$ requires C, 58·35; H, 4·3; $2OCH_3$, 12·1; $4CH_3CO$, 33·5%.)

Hydrolysis of dimethylasperthecin tetraacetate. Dimethylasperthecin tetraacetate (0·20 g.) was boiled under reflux with methanol (15 ml.) containing 3% (v/v) conc. H_2SO_4. The hydrolysis proceeded in a similar manner to that of hexaacetylasperthecin (see this page); after 1·5 hr. the mixture was cooled and the precipitate of bright red needles was collected (0·12 g.), washed with methanol and then water. When crystallized from dioxan this product formed bright red thin blades, identical in all respects with dimethylasperthecin; m.p. and mixed m.p. 236–237°.

Degradation of asperthecin

By zinc dust distillation. Isolation of 2-methylanthracene. An intimate mixture of asperthecin (0·20 g.) and electrolytic Zn dust (15 g.) was heated in portions of approx. 1 g. in special glass tubes as described by Kögl & Postowsky (1924). The pale yellow crystalline distillates were dissolved in ether, dried over KOH and the solution was evaporated. The residue (10 mg.) was sublimed *in vacuo* at 110° and crystallized twice from ethanol to yield almost colourless leaflets, m.p. 195–197°. A mixture with authentic 2-methylanthracene, m.p. 203–204°, melted at 201–202°. The ultraviolet absorption spectrum of the product was compared with that of authentic 2-methylanthracene, with the following results. Zn dust product: light absorption max., 253·5, 310, 326·5, 342, 359 and 379 mμ. ($\log \epsilon$ 5·18, 3·30, 3·53, 3·75, 3·89 and 3·83 respectively). Found on authentic 2-methylanthracene: light absorption max., 254, —, 326·5, 342, 359·5 and 379 mμ. ($\log \epsilon$ 5·20, —, 3·42, 3·69, 3·81 and 3·76 respectively). There was no maximum at 310 mμ.

With HI and red phosphorus. Isolation of Frangula *emodin.* A mixture of asperthecin (0·50 g.), red P (0·50 g.), glacial acetic acid (15 ml.) and HI (3 ml., sp.gr., 1·7) was boiled under reflux for 5 hr. The brown mixture was cooled, poured into water (500 ml.) and decolorized with a little $NaHSO_3$. The resulting purple-brown precipitate was collected, washed with water, dried and extracted with boiling glacial acetic acid (50, 20, 20 ml.). Concentration of the combined brown extracts to 15 ml. yielded a light brown micro-crystalline product (0·28 g.) which gave the colour reactions of an anthranol.

The crude anthranol (0·26 g.) was dissolved in glacial acetic acid (20 ml.) by warming, and to the cooled solution was added a solution of CrO_3 (0·3 g.) in glacial acetic acid

(15 ml.). After standing for 30 min. at room temp. the brown mixture was diluted with much water and extracted 3 times with ether. The orange ethereal extracts were freed from acetic acid and a trace of coloured material by shaking repeatedly with aqueous $NaHCO_3$, and were then washed with water and evaporated to dryness. The brown-orange residue (0·10 g.) was sublimed in high vacuum at 180° and the orange sublimate was crystallized from toluene (25 ml.) giving orange needles, m.p. 256° alone or mixed with authentic *Frangula* emodin, m.p. 256°. (Found, on sample sublimed at 180° in high vacuum: C, 66·8, 66·9; H, 3·8, 3·7. Calc. for $C_{15}H_{10}O_5$: C, 66·7; H, 3·7%.) The product and authentic emodin gave indistinguishable solutions in N-Na_2CO_3 (slightly bluish red) and cold conc. H_2SO_4 (red). For confirmation, a portion of the product was converted into the acetate, which crystallized from ethanol as yellow needles, m.p. 196–197°, not depressed on admixture with authentic triacetylemodin, m.p. 197–198°.

Tests for fluorescence in asperthecin solutions

Raistrick *et al.* (1934, p. 567) showed that acetic acid solutions of polyhydroxyanthraquinones which are derivatives of quinizarin (1:4-dihydroxyanthraquinone) are strongly fluorescent in daylight, whereas those which do not contain the 1:4-dihydroxy grouping are not fluorescent. Hence, fluorescence was regarded as being characteristic of quinizarin derivatives. Because of the importance of this test in deducing the structure of asperthecin, solutions of asperthecin in glacial acetic acid and in cold conc. H_2SO_4 were examined for fluorescence on a number of occasions. No fluorescence in daylight was ever observed, although a number of other mould colouring matters, known to be derivatives of quinizarin, all showed strong fluorescence when compared side by side with asperthecin. Moreover, when examined in ultraviolet light, these solutions fluoresced brilliantly, whereas asperthecin showed only a pale dull red fluorescence in H_2SO_4 and a pale cream fluorescence in glacial acetic acid. Both these solutions were similar in colour to, but slightly less intense than, those shown by emodin (4:5:7-trihydroxy-2-methylanthraquinone, II) when examined under the same conditions. Further, when a little solid magnesium acetate was added to an ethanolic solution of asperthecin a strong purple colour was formed but without fluorescence (Shibata, 1941, p. 105). In the same test a number of quinizarin derivatives gave magenta coloured solutions which had a red fluorescence.

Dyeing tests with asperthecin and dimethylasperthecin

White wool was mordanted with chromium, tin or aluminium, using respectively $K_2Cr_2O_7$, $SnCl_2$ or aluminium acetate, as described by Cain & Thorpe (1913). A solution of the substance under test was made in dioxan (10 mg./ml.). This solution (0·3 ml.) was added to water (5 ml.) with shaking, and the mordanted wool (0·1 g. approx.) was immersed in it immediately. The tube was placed in a bath of cold water which was gradually heated to boiling and kept there for 1 hr. The dyed wool was then washed successively in running water, hot soap solution and finally in running water. The following results were obtained. Asperthecin, intense coloration of the fibre with all three mordants; Cr, indigo blue; Al, deep violet; Sn, dull red. Dimethylasperthecin, feeble dyeing of the fibre with all three mordants; Cr, pale blue; Al, pale violet; Sn, flesh pink.

When compared with alizarin, asperthecin gave colours on wool of equal intensity and brilliance, but for each mordant the colour was bluer than that of alizarin. On the other hand, the colours given by dimethylasperthecin were equal in intensity only to those given by a hydroxyanthraquinone which is not a mordant dyestuff, e.g. in this case, islandicin.

THE COLOURING MATTER OF *A. QUADRILINEATUS* STRAIN S.M. 297

Preliminary experiments, for which we are indebted to Mrs S. Marcus, indicated that freshly harvested mycelia of this strain gave colour reactions, particularly with cold conc. H_2SO_4, which were significantly different from those given by strain S.M. 200 (*q.v.*). Thus, pressed portions of the mycelium of strain S.M. 297 grown for 21 days on either Czapek–Dox or Raulin–Thom solutions, gave an immediate, intense prussian blue colour when immersed in cold conc. H_2SO_4 (strain S.M. 200 gives a deep purple colour) and intense purple-violet colours in N-Na_2CO_3 and aqueous N-NaOH which, particularly with the latter reagent, faded quickly. Extraction of the mycelium with cold organic solvents also gave solutions which were much redder and less orange than corresponding extracts of strain S.M. 200. Finally, the culture filtrate of strain S.M. 297, grown for 33 days on Raulin–Thom solution, quickly gave a heavy dark orange precipitate (0·35 g./flask) with Brady's reagent (0·32% 2:4-dinitrophenylhydrazine in aqueous 2N-HCl). The precipitate, which is being investigated in this laboratory, is insoluble in $NaHCO_3$, gives a purple-red Neuberg reaction and crystallizes from acetone as orange-red needles, m.p. 235° (decomp.). Only a small precipitate is slowly given by strain S.M. 200 under comparable conditions.

All attempts to isolate, in a pure crystalline condition, the colouring matter giving the prussian blue H_2SO_4 colour have so far failed, owing, we believe, to the ease with which it is converted into asperthecin. In attempting to isolate and purify it, however, batches of 1 l. conical flasks, each containing 350 ml. of Czapek–Dox solution (glucose, 50·0 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4$, $7H_2O$, 0·5 g.; $FeSO_4$, $7H_2O$, 0·01 g.; distilled water, 1000 ml.), were sterilized, inoculated with *A. quadrilineatus* strain S.M. 297 and incubated at 24°. At the end of the incubation period the mycelium was folded, pale brownish grey on the surface with a dark brownish purple reverse. The pigments present in the harvested mould were extracted with ethanol from the acidified and disintegrated mycelium exactly as is described on p. 481 for the isolation of asperthecin. The results obtained in two typical batches are summarized in the lower part of Table 1. Since the average weight of dry mycelium was approximately 6·5 g./flask, the yields of colouring matter obtained correspond to 1·0% of the dry weight of the mycelium for batch 1 (22 flasks) and 1·4% for batch 2 (21 flasks). The crude colouring matter obtained was a light brown, mainly crystalline solid and all batches of it showed the intense blue colour with cold conc. H_2SO_4 which is characteristic of the colour given by the whole mycelium. In other respects, however, the fractions behaved in the same way as similar fractions isolated from *A. quadrilineatus* strain S.M. 200.

The following attempts have been made to purify the crude crystalline pigment (Table 1, column 5) and to isolate from it the colouring matter giving the prussian blue H_2SO_4 colour.

By direct crystallization. A portion was crystallized from dioxan. As purification proceeded, the H_2SO_4 reaction of the crystals obtained became more and more purple. No material giving a blue H_2SO_4 colour could be obtained from the mother liquors.

By acetylation. A portion (0·30 g.) was acetylated with acetic anhydride and conc. H_2SO_4 as described for the crude pigment from *A. quadrilineatus* strain S.M. 200 (see p. 481). Yellow crystals separated from the reaction mixture and were collected (0·21 g.). A further quantity of amorphous yellow acetate (0·23 g.) was obtained by shaking the filtrate with water. Neither fraction gave the pure blue H_2SO_4 colour given by the starting material; the crystalline fraction gave a blue-purple and the amorphous fraction a purple-blue.

Purification of the crystalline fraction by crystallization first from glacial acetic acid and then from ethanol yielded pale yellow needles, m.p. 228°, alone or mixed with authentic hexaacetylasperthecin, m.p. 228°, prepared from *A. quadrilineatus* strain S.M. 200. (Found, on material dried in high vacuum at 90°: C, 56·6; H, 4·0. Calc. for $C_{27}H_{22}O_{14}$: C, 56·8; H, 3·9%.) The product gave a purple colour in cold conc. H_2SO_4 which was indistinguishable from that given by hexaacetylasperthecin in the same reagent. The purified acetate, m.p. 228°, was hydrolysed by boiling in methanol–H_2SO_4 (see p. 482), and the resulting crystalline product was recrystallized from dioxan to yield asperthecin as clusters of bright red needles. (Found, on material dried to constant weight at 130°: C, 56·5; H, 3·4. Calc. for $C_{15}H_{10}O_8$: C, 56·6; H, 3·2%.) The product and authentic asperthecin from *A. quadrilineatus* strain S.M. 200 were indistinguishable in their reactions towards the following reagents: cold conc. H_2SO_4, purple; cold conc. HNO_3, blue; 0·5 N-$NaHCO_3$, stable, purple-red coloured solution; 0·5 N-Na_2CO_3, purple-red solution, fading overnight; 0·5 N-NaOH, purple solution, fading in a few seconds; ethanolic $FeCl_3$, purple.

Crystallization of the amorphous acetate failed to yield any product giving a blue H_2SO_4 colour, but yielded only impure specimens of hexaacetylasperthecin.

By chromatography. A solution of the crude pigment in acetone was passed through a column of $CaCO_3$, which was then well washed with the same solvent. Two main bands—an upper and a lower—were formed and were separated mechanically, dissolved in dilute HCl and ice, and extracted with ether. The upper band, violet in colour, gave a reddish orange ether extract, which on evaporation gave an amorphous dirty brown solid (H_2SO_4 blue) from which, on acetylation, impure hexaacetylasperthecin (H_2SO_4 purple) was obtained. The lower band, violet-red in colour, gave an orange ether extract from which, on concentration, needles of asperthecin (H_2SO_4 purple) separated.

The instability of the pigment giving the blue H_2SO_4 colour was further demonstrated as follows. A little of the ethanolic extract of the mycelium of *A. quadrilineatus* strain S.M. 297 (H_2SO_4 blue) was diluted with ether, and the reddish solution was kept on the bench for 1 week. At the end of this time, the colour of the solution had changed to orange, and a portion of it, when dried and dissolved in cold conc. H_2SO_4, gave a purple solution. The conclusion therefore seems inevitable that the colouring matter of *A. quadrilineatus* strain S.M. 297, which gives the prussian blue H_2SO_4 colour, is very unstable and is readily converted, possibly by tautomeric change, into asperthecin.

SUMMARY

1. The purple-red colouring matter of the perithecia and ascopores of two different strains of *Aspergillus quadrilineatus* Thom & Raper has been isolated in pure crystalline form and has been named asperthecin.

2. Evidence is presented that asperthecin is the colouring matter which is characteristic of the purple-red perithecia and ascopores of a number of other species in the *Aspergillus nidulans* group.

3. Asperthecin, $C_{15}H_{10}O_8$, forms chestnut-brown needles, m.p. above 370°.

4. A number of functional derivatives and degradation products of asperthecin are described.

5. Asperthecin is believed to be either 3:4:5:6:7- or 1:4:5:6:7-pentahydroxy-2-hydroxymethylanthraquinone.

For the determination and interpretation of the infrared absorption spectra of asperthecin and many other polyhydroxyanthraquinones we wish to thank Dr Gunhild Aulin-Erdtman, Royal Institute of Technology, and Dr K. E. Almin, Swedish Forest Products Research Laboratory, Stockholm; Mr J. F. Grove and Dr L. A. Duncanson, Butterwick Research Laboratories of Imperial Chemical Industries Ltd., Welwyn, Herts; Mr J. W. Ryde, F.R.S., and Dr B. S. Cooper, Research Laboratories, General Electric Co. Ltd., Wembley; and Professor Shoji Shibata, Tokyo University, Japan.

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APPENDIX

Ultraviolet Absorption Spectra of some Polyhydroxyanthraquinones

By J. H. BIRKINSHAW
Department of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

In an endeavour to obtain guidance as to the location of the hydroxyl groups of polyhydroxyanthraquinones isolated from natural sources, and, in particular, of asperthecin, the ultraviolet absorption spectra of some of the natural and synthetic hydroxyanthraquinones of known constitution were examined in ethanolic solution and compared. Briggs, Nicholls & Paterson (1952) had made such determinations on compounds containing up to two free α-hydroxyl groups, but it appeared desirable to extend these observations to compounds of higher α-hydroxyl content. The wavelength and molar extinction coefficients of the maxima of the curves obtained are shown in Table 1 (p. 486). The absorption spectra were determined by means of a Hilger and Watts 'Uvispek' spectrophotometer.

Nearly all the curves show two major bands or groups of maxima (group *A*, lower λ and group *B*, higher λ) separated by a deep trough in the region 300–400 mμ. Chrysophanol and emodin (and ω-hydroxyemodin, the curve for which is almost indistinguishable from that of emodin) each containing two α-hydroxyl groups, are similar spectrographically; the additional hydroxyl group in the β- position present in emodin has only a small effect on the λ-values of the maxima, although the log ε values of the peaks in group *A* are considerably altered. In anthragallol (two β-, one α-OH) all the maxima occur at lower λ and the 285–290 band is intensified, relative to the other maxima of group *A*.

Islandicin and helminthosporin (three α-OH), which differ only in the position of the methyl group, show a general displacement of the bands towards the longer wavelengths only small in group *A*, but very marked in group *B*, with the appearance of fine structure in the latter group, three or four maxima being revealed in this range. The addition of a further α-hydroxyl group, represented by cynodontin (four α-OH) produces even further bathochromic displacement in group *B*, whereas in catenarin (three α-, one β-OH) the additional β-hydroxyl group has little effect on group *B* but seems to enhance the fine structure of group *A*, where there are now four clearly marked maxima as in emodin.

Rufigallic acid (two α, four β-OH) has one feature which distinguishes it from all the other compounds examined, namely a peak in the 350 mμ. region (anthragallol has a slight inflexion at this point). It is also characterized by a very high maximum at 295 mμ., even higher than anthragallol, although the general form of the curve is similar in the two cases, particularly in the group *B* region.

These results confirm the observations of Briggs *et al.* (1952) that the major band in the region from 350 mμ. upward is greatly affected by the number of α-hydroxyl groups, the presence of β-hydroxyl groups having little effect. It seems, however, that the number and position of the maxima in the lower wave band region (below 350 mμ.) is considerably influenced by the β-hydroxyl groups.

If the absorption spectrum of asperthecin is compared with the curves for those substances of known constitution which have been examined, it is evident that the closest resemblance is presented by the spectrum of catenarin. The resemblance is particularly marked in the group *B* portion, which suggests that three and not more than three α-hydroxyl groups are present in asperthecin. The differences in the group *A* portion of the curves could well be accounted for by the additional nuclear hydroxyl present in asperthecin, which if the above deduction is correct, must be in the β- position.

SUMMARY

1. The ultraviolet absorption spectra of nine natural and synthetic polyhydroxyanthraquinones of known structure have been determined.

2. From a comparison of these spectra with that of asperthecin it is concluded that the molecule of asperthecin probably has three α-hydroxyl groups in its structure.

The author thanks the Central Research Fund Committee of London University for a grant for purchase of a spectrophotometer.

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Table 1. *Ultraviolet spectra of polyhydroxyanthraquinones in ethanol*

Substituted anthraquinone	λ 210–240		λ 240–265		λ 265–287		λ 287–320		λ 320–480		λ 480–520		λ 520–560	
	mμ.	log ε	mμ.	log ε	mμ.	log ε	mμ.	log ε	mμ.	log ε	mμ.	log ε	mμ.	log ε
Chrysophanol (2-methyl-4:5-dihydroxy)	225	4·56	255	4·34	277·5	4·04	287·5	4·07	430	4·08	—	—	—	—
Emodin (2-methyl-4:5:7-trihydroxy)	222	4·55	252	4·26	265	4·27	289	4·34	437	4·10	—	—	520–30*	2·38
ω-Hydroxyemodin (2-hydroxymethyl-4:5:7-trihydroxy)	222·5	4·46	250·5	4·21	266	4·21	289·5	4·26	436	4·04	—	—	510–20*	2·92
Anthragallol (1:2:3-trihydroxy)	213	4·25	241 245	4·28 4·30	—	—	287	4·49	414	3·81	—	—	—	—
Islandicin (2-methyl-1:4:5-trihydroxy)	232	4·52	252·5	4·28	—	—	289	3·86	390–402* 466–70*	3·32 3·98	492 513	4·09 3·96	527	3·92
Helminthosporin (2-methyl-4:5:8-trihydroxy)	231	4·53	255	4·15	—	—	289	3·79	405–15*	3·85	480 490 510	3·99 4·01 3·89	525	3·81
Catenarin (2-methyl-1:4:5:7-tetrahydroxy)	231	4·51	255	4·23	280	4·24	298	4·03	—	—	488·5 508	4·16 4·06	515–525*	3·99
Cynodontin (2-methyl-1:4:5:8-tetrahydroxy)	221 237·5	4·22 4·32	—	—	—	—	296	3·63	—	—	518	3·90	545 558	3·88 3·94
Rufigallic acid (1:2:3:5:6:7-hexahydroxy)	213 222	4·21 4·18	258	3·85	—	—	295	4·64	349 438	3·92 3·92	—	—	—	—
Asperthecin	237·5	4·41	262·5	4·49	286·5	4·27	318	4·02	—	—	484 510	4·24 4·23	545–55*	3·66

* Inflexion.

Free and Fixed Glycogen in Rat Muscle

By ADRIENNE J. M. KITS VAN HEIJNINGEN and A. KEMP
Pharmaco-Therapeutic Laboratory, University of Amsterdam, Amsterdam, Holland

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It has been known for many years that part of the tissue glycogen remains undissolved when tissues are extracted with trichloroacetic acid (TCA). This fact led Nerking (1900) and Willstätter & Rhodewald (1934) to conclude that tissue glycogen appears in two forms: an acid-extractable or free form and an acid-non-extractable or protein-fixed form. According to Pflüger (1903) and Loeschke (1904) this protein-fixed fraction is produced by the enclosure of part of the glycogen by the proteins in the course of their precipitation, and should be considered an artifact. It has never been settled satisfactorily since, whether the glycogen–protein complexes are artifacts or whether they constitute a physiological entity.

In a previous paper (Kemp & Kits van Heijningen, 1954) on a new micro-method for the determination of glycogen it was shown that the fixed glycogen can be extracted from tissues with TCA at 100°, and it was pointed out that the method could be easily adapted to the study of these two glycogen fractions in tissues. In the present work we have used this method to approach in two ways the question of the artificial or physiological nature of the protein-fixed glycogen in muscle. (i) It was tested whether a physiological process, i.e. the performance of work by a muscle, would affect the two glycogen fractions differently, thus altering their ratio. (ii) It was investigated whether different methods of glycogen extraction would yield the same values for both fractions respectively.

EXPERIMENTAL

Determination of free and fixed glycogen. Glycogen was extracted with a 5% (w/v) TCA solution containing 0·1% of silver sulphate and determined by means of a colour reaction (Mendel & Bauch, 1926) which occurs when glycogen is heated with concentrated sulphuric acid (Mendel, Kemp & Myers, 1954; Kemp & Kits van Heijningen, 1954). In order to separate the two fractions the extraction of tissue samples with the TCA reagent was performed in two stages:

Stage 1. The tissue sample was homogenized in the TCA reagent and subsequently centrifuged at 3000 rev./min. The clear supernatant contains all the free glycogen plus glucose and glucose 1-phosphate. The glucose and glucose 1-phosphate were determined in the 80% (v/v) methanol extract of a separate sample and the value found was subtracted from the glycogen plus glucose value (for details see Kemp & Kits van Heijningen, 1954).

Stage 2. The tissue residue was then washed once with the TCA reagent, heated for 15 min. at 100° with another 5 ml. of the reagent and centrifuged. This supernatant contains the fixed glycogen.

Muscular work. Female rats of a cross-bred Wistar strain, weighing 250–300 g. were used. The animals were anaesthetized with sodium pentobarbitone 45 mg./kg. intraperitoneally. If necessary another 2 or 3 mg. of pentobarbitone were given in the course of the experiment. One gastrocnemius muscle was freed from surrounding tissues, the achilles tendon was cut and connected to a lever weighted with 10 g. The sciatic nerve was freed, dissected and, with the aid of a silk thread attached to the end, placed over a pair of needle electrodes from a square wave stimulation apparatus. Stimuli of 0·8 mA strength and 20 msec. duration were applied every second. At the end of the stimulation period the glycogen in both gastrocnemius muscles was determined, the unstimulated one serving as control.

Extraction experiments. Two different methods of extraction were compared, namely the TCA extraction described above and an extraction with distilled water containing 0·02 M ethylenediaminetetraacetic acid (EDTA) (Versene) to prevent glycogenolysis. According to Gross (1953) the enzymic function of myosin-ATPase can be inhibited by removing Ca ions with the aid of EDTA, a chelating agent. ATPase activity is essential to provide the inorganic phosphate required for the phosphorylysis of glycogen. It was expected, therefore, that EDTA, in a concentration sufficient to inhibit ATPase activity, would inhibit glycogenolysis. In preliminary experiments a satisfactory inhibition of glycogenolysis in muscle was obtained by using a concentration of 0·02 M EDTA. The animals were killed by a blow on the head, and one gastrocnemius muscle was removed immediately.

Muscle samples of 250–500 mg. were weighed quickly on a torsion balance and ground with chloride-free quartz sand in mortars containing 5 ml. of the EDTA solution. The brei was transferred quantitatively into a centrifuge tube with the aid of another 3 ml. of EDTA solution and centrifuged subsequently at 3000 rev./min. for 5 min. The clear supernatant was decanted into another centrifuge tube and deproteinized by adding 2 ml. of a 20% (w/v) solution of metaphosphoric acid containing 400 mg./100 ml. of silver sulphate or by adding 2 ml. of 20% (w/v) TCA containing 400 mg./100 ml. of silver sulphate. The precipitate thus formed in the EDTA extract (VE precipitate) was centrifuged down, the clear supernatant decanted and its glycogen content determined. Up to this point the experiments were carried out in the cold room. The tissue residue and the VE precipitate were washed once with the ordinary TCA reagent, and the fixed glycogen was extracted and determined as described above.

RESULTS

As can be seen from Fig. 1, computed from control determinations over a period of a year, the values for both fractions as well as their ratios vary greatly from one animal to another. Even in a litter of seven animals, which were kept for 3 months under carefully controlled constant conditions, a great variation in the free : fixed glycogen ratio was found (Table 1).

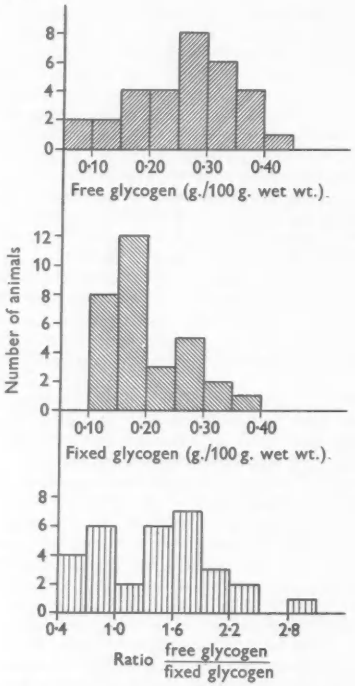
Number of animals
Free glycogen (g./100 g. wet wt.)
Fixed glycogen (g./100 g. wet wt.)
Ratio free glycogen / fixed glycogen
0·10 0·20 0·30 0·40
0·4 1·0 1·6 2·2 2·8

Fig. 1. Frequency distribution of free and fixed glycogen contents, and the ratio free : fixed glycogen in the gastrocnemius muscles of thirty-one normally fed rats.

In Table 2 a comparison is made between the values for both glycogen fractions in the left and right gastrocnemius muscle of the same animal. As could be anticipated from the results found by Cramer (1888) in other animal species, the differences do not exceed those between different pieces of the same muscle. From Table 3 it appears that a lapse of time of 3 hr. under anaesthesia, between the removal of left and right gastrocnemius muscle, does not influence the glycogen content of the muscle which is removed last. Experiments were therefore designed in such a way that each animal could serve as its own control.

In Table 4 the results of the stimulation experiments are given. In the last two experiments, stimulation was carried on until the muscle was no longer able to lift the weighted lever. To shorten the duration of the experiments the weight on the lever was increased as indicated in the table.

The glucose and glucose 1-phosphate concentrations were determined in the experimental and in the control muscles. As it appeared that the content of the experimental and control samples was the same, their mean value was calculated and given in the table.

Table 1. *Free and fixed glycogen content in the gastrocnemius muscles of seven rats of one litter*

The animals were kept for 3 months under constant conditions. Mean values of two determinations are given. Glycogen values are expressed in terms of glucose equivalents.

Rat no.	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)	Ratio: free glycogen / fixed glycogen
1	0·20	0·16	1·25
2	0·23	0·14	1·64
3	0·25	0·16	1·56
4	0·32	0·17	1·88
5	0·26	0·13	2·0
6	0·28	0·13	2·15
7	0·22	0·13	1·69
		Mean value ± S.E.	1·74 ± 0·26

Table 2. *Comparison of free and fixed glycogen content in left and right gastrocnemius muscles of single rats*

Mean values are given ± S.E. Glycogen values are expressed in terms of glucose equivalents.

Samples of left muscle				Samples of right muscle			
Wt. of muscle samples (mg.)	No. of determinations	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)	Wt. of muscle samples (mg.)	No. of determinations	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)
207–230	5	0·35 ± 0·03	0·19 ± 0·02	186–252	5	0·39 ± 0·04	0·18 ± 0·02
179–211	4	0·33 ± 0·03	0·17 ± 0·00	186–270	4	0·29 ± 0·02	0·19 ± 0·01
171–229	4	0·33 ± 0·02	0·11 ± 0·01	139–182	4	0·33 ± 0·02	0·13 ± 0·01
144–206	4	0·34 ± 0·01	0·19 ± 0·01	154–254	3	0·35 ± 0·01	0·19 ± 0·03
26–42	3	0·21 ± 0·01	0·30 ± 0·01	24–38	3	0·21 ± 0·02	0·30 ± 0·01
182–239	4	0·34 ± 0·01	0·18 ± 0·01	180–211	4	0·39 ± 0·01	0·18 ± 0·01
172–198	4	0·29 ± 0·01	0·16 ± 0·01	173–204	4	0·31 ± 0·01	0·16 ± 0·01

Table 3. *Comparison of free and fixed glycogen content of left and right gastrocnemius muscles of single rats*

The animals were anaesthetized with sodium pentobarbitone. The left muscle was removed after the onset of anaesthesia and the right one 3 hr. later. Mean values of three determinations are given ± S.E. Glycogen values are expressed in terms of glucose equivalents.

Samples of left muscle			Samples of right muscle		
Wt. of muscle samples (mg.)	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)	Wt. of muscle samples (mg.)	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)
59–74	0·42 ± 0·03	0·21 ± 0·004	51–88	0·42 ± 0·02	0·28 ± 0·02
63–106	0·43 ± 0·02	0·23 ± 0·02	74–86	0·49 ± 0·02	0·23 ± 0·01
54–100	0·52 ± 0·003	0·23 ± 0·01	76–95	0·56 ± 0·03	0·24 ± 0·01
53–86	0·35 ± 0·002	0·12 ± 0·01	45–71	0·30 ± 0·003	0·11 ± 0·02
39–83	0·42 ± 0·02	0·17 ± 0·02	44–56	0·35 ± 0·01	0·20 ± 0·02
47–101	0·45 ± 0·04	0·17 ± 0·01	59–86	0·48 ± 0·02	0·20 ± 0·02

In several experiments blood sugar values were determined according to Mendel *et al.* (1954) (cf. Mendel & Hoogland, 1950), before the onset of anaesthesia, after the preparation of the muscle for stimulation, and at the end of the stimulation period. Anaesthesia and preparing the muscle for stimulation did not affect the blood sugar markedly. Only in the last experiment was a considerable decrease in blood sugar found at the end of the stimulation period, namely from 72 mg./100 ml. before anaesthesia to 47 mg./100 ml. at the end of the experiment. In this experiment, liver samples were taken at the end of the stimulation period, and their glycogen content determined. The values were 0·11 and 0·09 g./100 g. wet wt. for the free fraction, and 0·08 and 0·06 g./100 g. wet wt. for the fixed glycogen. This very low glycogen content might explain the marked fall in blood sugar found at the end of this experiment.

In Table 5 the results of the extraction with water containing EDTA are compared with those of the TCA extraction. In some of the experiments the EDTA extracts were deproteinized with metaphosphoric acid instead of TCA. It appears that a small part of the fixed glycogen dissolves in the distilled water. This part is recovered by the hot TCA extraction of the VE precipitate. The extractions with cold TCA and with distilled water yield the same values for the free glycogen fraction. The sum of the values found by hot extraction with TCA of the VE precipitate and the residue of the aqueous extracts is in good agreement with the values found for the fixed fraction by the TCA extraction method.

DISCUSSION

No constant ratio could be demonstrated between the free and fixed glycogen in the gastrocnemius muscle of the rat. On this point our results are at variance with those of Bloom, Lewis, Schumpert & Shen (1951), who postulate a constant ratio of 1·22. However, the free and fixed glycogen contents of the right and left gastrocnemius muscle of the same rat were found to be identical.

As appears from Table 4 the two fractions behave differently under the influence of work performance, and consequently their ratio changes. This is in agreement with the results found by Bloom & Knowlton (1953) in similar stimulation experiments, though their methods of stimulation and estimation are essentially different.

On the other hand, two different methods of extraction, one of which is not likely to precipitate proteins that might enclose the glycogen, yield the same values for the free and fixed glycogen fractions respectively. It seems therefore that the fixed glycogen fraction which is found in muscle as well as in liver and other tissues is not an artifact, but a physiological entity. Presumably one of the two fractions represents the stored glycogen, whereas the other constitutes the 'active' form. However, our experiments do not answer the question which function should be ascribed to which fraction.

SUMMARY

1. The occurrence of free and fixed glycogen in the gastrocnemius muscle of the rat was studied.

2. Considerable individual differences were observed in the ratio, free:fixed glycogen. Identical values, however, were found for free and fixed glycogen respectively, in the right and left gastrocnemius muscle of the rat.

3. Performance of work alters the ratio free: fixed glycogen in the gastrocnemius muscle.

4. Two different methods of extraction, i.e. with trichloroacetic acid and with water containing ethylenediaminetetraacetic acid to prevent glycogenolysis, yield the same values for the two glycogen fractions respectively.

5. It is concluded that the fixed glycogen is probably a physiological entity and not an artifact.

The authors are indebted to Professor B. Mendel for much valuable discussion.

Table 4. *Influence of electrical stimulation on free and fixed glycogen in gastrocnemius muscles of rats*

In each animal one muscle was stimulated via the nerve with a square wave current of 0·8 mA for 20 msec. every second. Mean values of three determinations are given ±S.E. In all cases except Expt. 3, free sugar was determined in the control and stimulated muscles, and the mean values were subtracted from those for free glycogen. Glycogen values are expressed in terms of glucose equivalents.

	Control muscles					Stimulated muscles					
Expt. no.	Wt. of muscle samples (mg.)	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)	Ratio: free / fixed glycogen	Free sugar (%)	Weight on lever (g.)	Duration of stimulation (min.)	Wt. of muscle (mg.)	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)	Ratio: free / fixed glycogen
1	61–236	0·31±0·01	0·15±0·01	2·07	0·06±0·02	10	2	40–226	0·23±0·04	0·16±0·02	1·44
2	55–236	0·29±0·03	0·14±0·004	2·07	0·06±0·01	10	2	62–231	0·21±0·04	0·13±0·01	1·61
3	153–252	0·31±0·04	0·19±0·02	1·63	—	10	4½	144–248	0·14±0·02	0·11±0·02	1·27
4	21–231	0·27±0·01	0·18±0·03	1·50	0·05±0·02	15	4½	28–246	0·16±0·03	0·14±0·02	1·14
5	58–240	0·19±0·01	0·16±0·01	1·19	0·09±0·01	10	9½	78–254	0·05±0·02	0·10±0·00	0·50
6	59–212	0·21±0·01	0·16±0·01	1·31	0·05±0·01	10	9½	98–180	0·06±0·02	0·05±0·01	1·20
7	66–260	0·31±0·01	0·17±0·01	1·82	0·04±0·01	10 20 30	93 10 10	113	32–235	0·06±0·01	0·07±0·003	0·86
8	39–275	0·33±0·005	0·17±0·004	1·94	0·00	10 20 30	90 10 32	132	58–243	0·04±0·01	0·05±0·01	0·80

Table 5. *Comparison of free and fixed glycogen content in the same rats' gastrocnemius muscle as obtained by extraction with TCA and* 0·02 M *EDTA*

The EDTA extracts were deproteinized with metaphosphoric acid (HPO_3) or TCA. Fixed glycogen was determined in the VE precipitate thus obtained, and in the tissue residues of TCA and EDTA extracts. The glycogen was determined colorimetrically as described by Kemp & Kits van Heijningen (1954). Mean values of three determinations are given ±S.E. When only two determinations were made the individual results are given. Glycogen values are expressed in terms of glucose equivalents.

	TCA extractions			Extraction with 0·02 N EDTA					
						Fixed glycogen content (%) in VE precipitate, precipitated with			
Rat no.	Wt. of muscle samples (mg.)	Free glycogen content (g./100 g. wet wt.)	Fixed glycogen content (g./100 g. wet wt.)	Wt. of muscle samples (mg.)	Free glycogen content (g./100 g. wet wt.)	TCA	HPO_3	Fixed glycogen content in residue (g./100 g. wet wt.)	Total fixed glycogen
1	130–174	0·20±0·01	0·16±0·01	389; 361	0·23; 0·22	0·05; 0·09	—	0·22; 0·19	0·27; 0·28
2	143–178	0·16±0·01	0·11±0·01	365–468	0·17±0·003	0·03±0·003	—	0·08±0·01	0·11±0·01
3	124–159	0·30; 0·27	0·19±0·02	283–494	0·26±0·03	0·06±0·01	—	0·11±0·01	0·17±0·01
4	137–175	0·28±0·01	0·21±0·01	445–493	0·29±0·02	0·08±0·003	—	0·20; 0·14	0·25
5	94–119	0·30±0·02	0·20±0·003	424–499	0·25±0·02	0·08; 0·08	—	0·16±0·02	0·24
6	109–162	0·23; 0·25	0·17±0·003	278–330	0·22±0·01	—	0·06±0·003	0·08±0·01	0·14±0·01
7	81–122	0·29±0·003	0·24±0·01	285–313	0·23±0·01	—	0·06±0·01	0·15±0·01	0·21±0·01
8	77– 95	0·25±0·01	0·18±0·01	320–357	0·23±0·01	—	0·04±0·01	0·11±0·01	0·15±0·01
9	102–128	0·25±0·02	0·19±0·01	368–497	0·29±0·02	—	0·06±0·02	0·11±0·02	0·17±0·03
10	68–108	0·26±0·01	0·16±0·01	299–451	0·23±0·01	—	0·08±0·01	0·09±0·03	0·17±0·03

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Studies in Carotenogenesis

14. CAROTENOID SYNTHESIS IN THE PHOTOSYNTHETIC BACTERIUM *RHODOPSEUDOMONAS SPHEROIDES*

By T. W. GOODWIN, D. G. LAND and H. G. OSMAN
Department of Biochemistry, The University, Liverpool 3

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The general features of the non-sulphur photosynthetic bacterium *Rhodopseudomonas spheroides* have been described by van Niel (1941). Under anaerobic conditions in the light, cultures are brown but, as noted by French (1940), on allowing them access to air their colour changes to purplish red. This is also the colour obtained in cultures grown aerobically from the start. French further observed that this colour change could not be brought about by cell-free extracts, nor could it be accelerated by the addition of oxidizing agents; furthermore, the reverse process, the conversion of red cultures into brown by changing from aerobic to anaerobic conditions, was not possible. From a study of the absorption spectra of the pigment extracts, French concluded that the colour change from brown to red was mainly due to the formation of a pigment with an absorption maximum at 550 mμ. (in carbon disulphide); he did not consider this pigment to be a carotenoid.

A more detailed study of the pigments of *R. spheroides* was later carried out by van Niel (1947) (the abbreviation *R.* will be used in this paper for *Rhodopseudomonas*). On chromatography of the pigments remaining after bacteriochlorophyll had been removed, two main carotenoid pigments, one yellow (*Y*) and one red (*R*) were obtained, neither of which could be identified with previously described carotenoids. Associated with *R* and *Y* were a number of minor components, most of which appeared to be *cis* isomers of the parent pigments. Van Niel (1947) also demonstrated that the brown → red (anaerobic → aerobic) change was characterized by the disappearance of *Y* accompanied by the appearance of an equivalent amount of *R*. This change also took place in the dark, although not to the same extent as in the light, but, in agreement with the observations of French (1940), it did not occur in cell-free extracts. Furthermore, van Niel (1947) confirmed that the reverse change red → brown could not be brought about.

The present investigation was undertaken to extend the observations of van Niel on carotenoid synthesis by *R. spheroides* in the light of present knowledge of carotenogenesis in general (Goodwin, 1953), and in another photosynthetic bacterium, *Rhodospirillum rubrum* (*Rsp. rubrum*) in particular (Goodwin & Osman, 1953, 1954).

EXPERIMENTAL

Cultures. The *R. spheroides* used throughout this investigation was a strain kindly supplied by Dr R. Y. Stanier, Department of Bacteriology, University of California, Berkeley. It was maintained in stab cultures on an agar medium. The liquid culture medium used was almost identical with that recommended by van Niel (1947): $(NH_4)_2SO_4$ (1·0 g.), $MgCl_2$ (0·5 g.), K_2HPO_4 (3·0 g.), sodium malate (3·0 g.), yeast extract (Oxoid) (1·0 g.), water 1000 ml. The final solution was adjusted to pH 6·8–7·0 with 6N-NaOH. The only difference from van Niel's medium is that 'Yeast autolysate (1·0 ml.)' (source unstated) has been replaced by the yeast extract. Inoculation of the liquid media was carried out with 1 ml. of a 5- to 6-day-old liquid culture. Anaerobic cultures were grown as previously described for *Rsp. rubrum* (Goodwin & Osman, 1953, 1954) in completely filled Pyrex bottles (100 ml.) fitted with ground-glass stoppers. Aerobic cultures (100 ml.) were grown in Erlenmeyer flasks (250 ml.). All cultures were shaken vigorously by hand twice a day. They were incubated at 30° either in the dark in a conventional electrically heated incubator or in the light in an all glass incubator illuminated on two sides with two banks of 4 × 60w incandescent lamps (Goodwin & Osman, 1953).

Washed suspensions were obtained using the same technique as described previously for *Rsp. rubrum* (Goodwin & Osman, 1954).

Analytical procedures

Dry weight determinations. Direct dry wt. determinations were carried out as described for *Rsp. rubrum*. (Goodwin & Osman, 1953). An attempt was made to relate the E (1 μ.) value of suspensions with their dry wt. as was done with *Rsp. rubrum*, but, contrary to the experience with this organism, the results obtained in preliminary experiments were not satisfactory and the direct determination of dry wt. was always carried out.

Extraction, separation and determination of the carotenoids. The extraction procedure used was that first described for *Rsp. rubrum* by Polgar, van Niel & Zechmeister (1944) and slightly modified by Goodwin & Osman (1953); it was further slightly revised to suit *R. spheroides*. The treatment of the methanol extract with aqueous KOH, to remove bacteriochlorophyll, was carried out for 2 min. at room temp. instead of at the b.p. This reduced to a minimum the *cis→trans* isomerism of the pigments, which in the case of Y and R can occur very easily. This method of extraction was, in our hands, more satisfactory than the method originally described for *R. spheroides* by van Niel (1947).

Chromatographic separation of the pigments. This was carried out according to the method of Goodwin & Osman (1953, 1954) using alumina (grade 'O': P. Spence and Co., Widnes) deactivated with methanol (Goodwin & Srisukh, 1949).

Quantitative determination of the pigments. The amounts of the two major components (Y) and (R) were calculated according to the equations of van Niel (1947) after measuring the E values of the solution of the mixed pigments in benzene at 500 and 530 mμ. This method is based on the assumption that there are no other pigments present; this is not strictly true but as the other pigments represent only a small percentage of the whole, the error caused by their presence is not large.

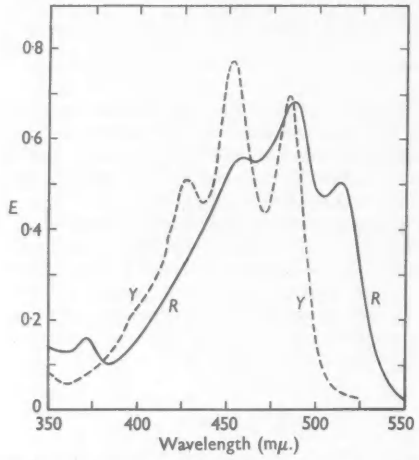

Fig. 1. Absorption spectrum in light petroleum (b.p. 40–60°) of pigments Y (– – –) and R (——).

RESULTS

The pigments present in Rhodopseudomonas spheroides

A number of extracts of anaerobic cultures were examined chromatographically on deactivated alumina. The separations obtained were always the same, but the relative amounts of the components tended to vary slightly from culture to culture. A typical separation is illustrated in Table 1 and corresponds closely with that obtained by van Niel (1947). Zones 2 and 5 are the most abundant fractions and are identical with pigments Y and R of van Niel. Their absorption spectra in light petroleum are recorded in Fig. 1. The remaining zones represent traces of pigment only, and could not be examined in great detail. Zone 1 was also noted by van Niel, who suggested that it might be flavorhodene (Karrer & Solmssen, 1936); the shape of its absorption spectrum appears, however, to

Table 1. *Chromatographic separation of* Rhodopseudomonas spheroides *carotenoids*

Adsorbent weak alumina; pigments listed in order of increasing adsorptive power. Anaerobic culture.

Zone	Description	Absorption spectra $\lambda_{max.}$ (mμ.) in light petroleum	Ether in light petroleum (% v/v) required to elute pigment from column
1	Yellow; trace	443, 470	2
2	Yellowish brown; major zone	428, 453, 485	5–10
3	Pink; trace	455, 483, 515	10
4	Yellow; trace	429, 452, 484	10
5	Pink; second most abundant zone	459, 488, 522	20
6	Trace of pink	462, 492, 525	20
7	Brownish pink; trace	Ill-defined maxima 482	25
8	Trace; yellowish brown	428, 453, 483	40
9 and 10	Trace of two separate dirty yellow zones with ill-defined maxima	—	60

preclude this possibility. Zone 4 is probably a *cis* isomer of *Y*. Zones 6–10 correspond to a zone described by van Niel as 'at least 6 small fractions of purplish, red and yellow adsorbates', and which he did not describe further. Zones 7, 9 and 10 have very indeterminate spectra and are probably oxidative artifacts; zone 6 might be spirilloxanthin (Polgar *et al.* 1944) it shows the correct chromatographic behaviour and absorption maxima, but the persistence of the bands is less than that of spirilloxanthin. Zone 8 has a spectrum identical in shape and position with *Y*. Because of its chromatographic properties, however, it must contain one hydroxyl group and is therefore probably monohydroxy *Y*. No colourless polyenes, such as phytoene or phytofluene, were detected.

Chromatography of the pigment extract of cultures of *R. spheroides* grown aerobically gave the same qualitative picture as that obtained with extracts of anaerobic cells. Quantitatively the relative amounts of *Y* and *R* were approximately reversed.

Pigment synthesis in growing cultures

In his investigations on *R. spheroides*, van Niel (1947) examined only the change in pigment distribution when mature cultures were transferred from anaerobic to aerobic conditions. He did not relate pigment production to growth (dry weight) of the bacterium, nor did he follow the change in pigment production with time. Table 2 gives the results of one of many experiments carried out to obtain this further information. Anaerobically, growth is complete after 5 days with the pigment concentration remaining almost constant throughout, the amount of *Y* present being just over twice that of *R*. Under aerobic conditions full growth is achieved at 4–5 days and in young cultures the concentrations of *R* and *Y* are almost exactly the reverse of those in anaerobic cultures. A further important difference between anaerobic and aerobic cultures is that whilst in the former the concentration of *Y* falls only slightly with increasing age, it drops much more rapidly in aerobic cultures; e.g. *Y* has almost

Table 2. *Dry weight and pigment production by* Rhodopseudomonas spheroides

Standard liquid medium; temp. 30°; amounts produced/100 ml. medium; each value represents the mean of at least two determinations; the variation between individual determinations is of the order ±2% for dry wt. and ±5% for pigments.

	Anaerobic cultures			Aerobic cultures		
Age of culture (days)	Dry wt. (mg.)	Pigment (mg./g. dry wt.) *R*	*Y*	Dry wt. (mg.)	Pigment (mg./g. dry wt.) *R*	*Y*
2	90	0·86	2·40	69	2·11	0·88
3	105	1·05	2·36	—	—	—
4	108	0·99	2·09	106	2·35	0·69
5	149	0·88	1·94	—	—	—
6	—	—	—	104	2·54	0·73
7	140	0·70	1·78	—	—	—
8	—	—	—	118	2·49	0·23
10	142	0·75	1·57	100	2·51	0·10

Table 3. *Change in pigment distribution on transferring* Rhodopseudomonas spheroides *from anaerobic to aerobic conditions*

Five-day cultures transferred for 24 hr. in light and 48 hr. in dark; except where stated otherwise. Temp. 28°; washed suspensions in 0·067 M-KH_2PO_4–K_2HPO_4 buffer.

Concentration of pigments (mg./g. dry wt.)								
Before transfer			After transfer: In dark			After transfer: In light		
R	*Y*	Total	*R*	*Y*	Total	*R*	*Y*	Total
Washed suspensions								
0·95	2·38	3·33	1·36	1·59	2·95	1·82	0·74	2·56
0·59	4·98	5·57	1·94	2·55	4·49	3·15	0·95	4·10
0·97	2·55	3·52	1·72	1·72	3·44	—	—	—
0·65	1·99	2·64	1·18	0·93	2·11	1·69	0·30	1·99
Unwashed suspensions								
1·37	4·31	5·68	(*a*)* 2·55	3·45	6·00	3·23	2·91	6·14
			(*b*)* 2·36	3·40	5·76	3·33	2·58	5·91

* (*a*) are 24 hr. transfers and (*b*) 48 hr. transfers.

completely disappeared from 8-day aerobic cultures. Anaerobic cultures are occasionally encountered with very high pigment concentration, especially of *Y* (see, for example, some of the experiments quoted in Table 3).

Observations on the anaerobic → aerobic pigment change

Four-day anaerobic cultures (100 ml.) were transferred to 250 ml. Erlenmeyer flasks either as such or after washing and resuspension in 0·067 M-K_2HPO_4–KH_2PO_4 buffer at pH 6·8. The results obtained (Table 3) confirm those of van Niel (1947) using unwashed cells, in demonstrating that admission of oxygen to anaerobic cultures in either darkness or light results in increased synthesis of *R* and the disappearance of *Y*.

In van Niel's experiments the amount of *Y* disappearing equalled the amount of *R* appearing; in the great majority of our experiments (including many not recorded) there was some loss of total pigment. An experiment recorded with unwashed cells is one of the few in which this did not occur.

Table 3 further shows that washed cells are also capable of bringing about this conversion and that conversion in the dark proceeds to a lesser extent than in the light. This is not just a matter of speed, for 48 hr. dark transfers differ very little from 24 hr. transfers.

Effect of diphenylamine

Diphenylamine, a specific inhibitor of carotenogenesis in a number of organisms (Goodwin, 1953), was tested on *R. spheroides*. Results under aerobic and anaerobic conditions (Table 4) were very similar. At a level of 1/35 000 diphenylamine completely inhibits growth, whilst at levels of 1/140 000 and 1/280 000, the only effect is that growth rate is slightly reduced. A concentration of 1/70 000, however, inhibits growth very considerably for 6–7 days and then, quite suddenly, the cultures produce normal growth. During the period when growth is inhibited, synthesis of pigment *R* is generally reduced slightly whilst that of *Y* is considerably reduced. Chromatography of the polyene extracts from young diphenylamine cultures did not reveal the presence of any of the more saturated polyenes (such as the phytofluene series).

Action of other inhibitors

Well-known metabolic inhibitors have been investigated for their effect on the anaerobic (yellow) → aerobic (red) pigment change both in light and in the dark and the effect compared with their action on growth. The results of a number of light experiments are recorded in Table 5; except for hydroxylamine, discussed below, results in the dark were exactly the same as in the light and are not recorded. The buffering capacity of the medium was adequate except in the presence of 10^{-2} M- and 10^{-1} M-KCN, when the pH rose to 9·7 and 10·6 respectively. Control experiments, however, showed that raising the pH to 10·6 had no effect on the pigment change.

As many of the experiments were carried out at different times, the results are expressed as the percentage of the concentration of pigment *R* compared with the control level (100 %); the disappearance of *Y* always parallels the increase in *R* and the *Y* values are therefore not recorded. The most important observation is the comparative immunity of the colour change to relatively

Table 4. *Effect of diphenylamine on growth and carotenoid synthesis by* Rhodopseudomonas spheroides

Amounts produced per 100 ml. culture, temp. 30°; 1:35000 completely inhibits growth both in aerobic and anaerobic conditions.

Concentration of diphenylamine	... 0			1:70 000			1:140 000			1:280 000		
		Pigment concn. (mg./g. dry wt.)			Pigment concn. (mg./g. dry wt.)			Pigment concn. (mg./g. dry wt.)			Pigment concn. (mg./g. dry wt.)	
Time (days)	Dry wt. (mg.)	*R*	*Y*	Dry wt. (mg.)	*R*	*Y*	Dry wt. (mg.)	*R*	*Y*	Dry wt. (mg.)	*R*	*Y*
					Anaerobic growth							
3	102	1·32	2·33	4·75	0·46	0·15	69	1·11	2·21	90	0·97	2·55
5	140	0·77	1·89	5·25	0·84	0·55	119	0·97	1·64	137	0·93	1·97
7	140	0·70	1·78	10·75	0·54	0·65	139	0·53	1·20	148	0·66	1·39
10	141	0·70	1·74	104	0·51	1·42	152	0·69	1·67	145	0·74	1·52
					Aerobic growth							
2	69	2·11	0·88	6·5	1·11	0·10	45	2·04	0·63	67	2·03	0·90
4	106	2·35	0·67	19·5	1·62	0·06	96	2·79	0·57	101	2·41	0·82
6	104	2·54	0·73	23	1·25	0·22	102	2·47	0·55	112	2·39	0·54
8	118	2·49	0·23	90	—	—	116	2·44	0·21	112	2·52	0·27
10	100	0·51	0·10	83	2·02	0·13	92	2·81	0·10	104	2·75	0·12

massive concentrations of the inhibitors; only with 10^{-2}M sulphanilamide and 10^{-1}M-KCN was marked inhibition achieved, and in these cases it was only because the cells had been killed. This was demonstrated by testing the viability of these cultures which showed inhibition by washing them and inoculating them into fresh medium. With 10^{-3}M hydroxylamine, inhibition has been consistently observed in the dark but not in light. A further important fact which emerges is that the inhibitors which, at certain concentrations, completely inhibit growth, never have any effect on the pigmentary changes.

DISCUSSION

R. spheroides is one of two photosynthetic bacteria in which carotenogenesis has been examined in detail, and it is instructive to compare it with the other organism, *Rsp. rubrum* (Goodwin & Osman, 1953, 1954); see Table 6. Although much more information on the general metabolism of these two organisms is required before the problem of carotenogenesis is finally solved, it appears that pigment synthesis is very different in each case. Especially important is the reaction of the two organisms to diphenylamine. With a concentration of 1/70 000 diphenylamine, young cultures of *R. spheroides* produce less pigment than do normal cultures; this is the same as in *Rsp. rubrum*, although no saturated polyenes are synthesized in *R. spheroides*. After 6 days treatment with diphenylamine, growth and pigment synthesis in *R. spheroides* suddenly become normal; no such phenomenon occurs with *Rsp. rubrum*.

Table 5. *The effect of various inhibitors on anaerobic growth and the anaerobic → aerobic colour change in* Rhodopseudomonas spheroides

Inhibitor	Concn.* (M)	Effect on colour change; amount of *R* produced compared with control (%)	Effect on growth
Arsenite	3×10^{-3}	112	Complete inhibition
2:4-Dinitrophenol	1×10^{-4}	85	Complete inhibition
Sulphanilamide	$1{\cdot}5 \times 10^{-2}$	88	Almost complete
Sulphanilamide	5×10^{-2}	No change (cells killed)	Complete inhibition
NH_2NH_2	10^{-2}	88	Complete inhibition
NH_2OH,HCl†	10^{-3}	99	Complete inhibition
NaN_3	10^{-2}	100	Complete inhibition‡
Iodoacetate	10^{-3}	103	Complete inhibition
KCN	10^{-3}	102	Complete inhibition
KCN	10^{-2}	84	
KCN	10^{-1}	31	
NaF	10^{-1}	91	Complete inhibition
Arsenate	10^{-1}	113	No effect
Malonate	4×10^{-2}	101	No effect
Diphenylamine	$8{\cdot}5 \times 10^{-5}$	108	(See Table 4)

* Only the highest concentrations examined are reported.

† This concentration of hydroxylamine inhibits the colour change in the dark; all other inhibitors have the same effect in light and darkness.

‡ Complete inhibition of growth was obtained at 10^{-3}M-NaN_3.

Table 6. *A comparison of carotenogenesis in* Rhodospirillum rubrum *and* Rhodopseudomonas spheroides

Experimental conditions	*Rsp. rubrum*	*R. spheroides*
Main pigments produced	One; Spirilloxanthin (*S*)	Two- *R* and *Y*
Effect of growth	Low concns. in young cultures	Same concn. throughout growth
Anaerobic culture	High concn. of *S*	High concn. of *Y*, low of *R*
Aerobic cultures	Lower concn. of *S*	Low concn. of *Y*, high of *R* Total same as under anaerobic conditions
Anaerobic washed cells suspended in phosphate buffer	No change in *S* content	Decrease in *Y* and increase in *R*
Action of diphenylamine (1/70000)	Inhibits *S* production; stimulates production of more saturated polyenes	Inhibits growth and pigment production in early stages, but after about 6–7 days both approach normality. No detectable synthesis of the more saturated precursors

Conversion of pigment Y into R

From his original investigations on *R. spheroides*, van Niel (1947) concluded that *Y* was converted into *R*. Although this conclusion may be correct, there are indications that another explanation is probably true. Table 2 shows that in growing aerobic cultures *Y* is decreasing although *R* is not increasing; that is, under one condition at least, *Y* undergoes a change other than a possible conversion into *R*. It is possible therefore, that in the anaerobic → aerobic change, synthesis of *R* from small molecules may suddenly be stimulated. This type of carotenogenesis has recently been observed in the green alga *Haematococcus pluvialis*; when the green cells of this alga are resuspended in phosphate buffer alone, large amounts of astaxanthin are synthesized *de novo* and not at the expense of the carotenoids already present (Goodwin & Jamikorn, 1954).

Furthermore, the change from *Y* to *R*, if it occurs, must be structurally complicated. The shifting of the main maxima of a carotenoid from 453 to 480 mμ., must essentially entail the production of 3–4 conjugated double bands, that is, the removal of 6–8 hydrogen atoms. In this case, that cannot be all, for a profound difference in the shape of the absorption spectra of *Y* and *R* exists (Fig. 1). The sharpness of the bands of *Y* suggest that it has a completely open-chain structure similar to that of lycopene (compare the definition of the bands of lycopene with that of β-carotene and of *Y*; Goodwin, 1952). The shape of the spectrum of *R*, in which the fine structure has almost completely disappeared, suggests either the appearance of one carbonyl group (the chromatographic behaviour of the pigment confirms this) or the ring closure of both ends of the open-chain compound. The addition of an hydroxyl or, possibly, a carbonyl group is required to account for the chromatographic behaviour of *R*. Thus, although the structures of *Y* and *R* are not yet known, it is obvious that they must differ considerably and that the postulated change $Y \rightarrow R$ cannot be a simple dehydrogenation.

If the change $Y \rightarrow R$ does take place, it is difficult to envisage it occurring in one step, and up to the present it has not been possible to detect any 'intermediates' in this postulated transformation.

SUMMARY

1. The presence of two major carotenoids (one yellow, *Y*, and one red, *R*), together with six minor components in *Rhodopseudomanas spheroides* has been confirmed. One of the minor fractions appears to be a monohydroxy derivative of *Y*. *Y* predominates in anaerobic and *R* in aerobic cultures. *Y* but not *R* disappears from aerobic cultures soon after they are fully grown.

2. The synthesis of *R* and the disappearance of *Y* when air is admitted to anaerobic cultures has been confirmed. The same reaction occurs in washed anaerobic cells resuspended in phosphate buffer.

3. Diphenylamine (1/70 000) inhibits growth and pigment synthesis for 5–6 days; then suddenly growth and carotenogenesis become normal. No saturated polyenes are synthesized on diphenylamine-containing media.

4. Many compounds which inhibit growth of *R. spheroides* do not, at the same concentrations, affect the yellow → red pigmentary change; this change is very insensitive to inhibitors.

5. Reasons are given for assuming that in the yellow → red change, pigment *Y* may not be converted into pigment *R*.

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Pyridoxal Phosphate as the Coenzyme of the Mammalian Decarboxylase for L-Cysteine Sulphinic and L-Cysteic Acids

By D. B. HOPE
Department of Pharmacology, University of Oxford

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Some time ago an enzyme was found in dog liver which decarboxylates L-cysteic acid (Blaschko, 1942). The product of the enzymic reaction is taurine (Blaschko, Datta & Harris, 1953). The enzyme has since been found in the rat and a number of other mammalian species.

It has always seemed likely that the enzyme was concerned with the *in vivo* formation of taurine, but this has never been fully established. It is known that in the pyridoxine-deficient rat the enzyme is lost (Blaschko, Carter, O'Brien & Sloane Stanley, 1948); at the same time taurine disappears from the rat's urine in which it is normally present (Blaschko *et al.* 1953). Recent work has made it likely that 2-aminoethane sulphinic acid (also called hypotaurine) is an intermediate in taurine synthesis (Bergeret & Chatagner, 1952; Bergeret, Chatagner & Fromageot, 1952; Awapara, 1953; Awapara & Wingo, 1953).

It has recently been shown by Chatagner, Tabechian & Bergeret (1954) that rat liver contains a decarboxylase for L-cysteine sulphinic acid and that the decarboxylation product is hypotaurine; this enzyme has also been shown to disappear in pyridoxine deficiency.

The experiments described in this paper make it likely that one and the same enzyme is responsible for the decarboxylation of both cysteic and cysteine sulphinic acids, a possibility already discussed by Awapara & Wingo (1953). In addition, evidence will be presented that the decarboxylase is a pyridoxal phosphate protein.

METHODS

L-Cysteine sulphinic acid was prepared from L-cystine by the method of Toennies & Lavine (1936). Calcium pyridoxal 5-phosphate was kindly given by Dr K. Folkers, Merck and Co., Rahway, New Jersey, U.S.A.

The rats used in the experiments on pyridoxine deficiency were of the hooded Lister strain obtained freshly weaned from the Laboratory Animals Bureau. The pyridoxine-deficient diet has already been described by Blaschko *et al.* (1953).

In some of the experiments rats were used which had been deficient in pyridoxine for only a few days. In these experiments, all the animals were put on the pyridoxine-supplemented diet for 12 days. In this period the animals doubled their body weight; the consequent increase in the size of the liver made it possible to use the material from one animal for the preparation of a sufficient amount of liver extract. Moreover, preliminary experiments had suggested that the enzymic activity in the livers of small rats weighing about 30 g. was low and somewhat variable. The animals and their diet were under the care of Miss M. A. Lewin, to whom I am grateful.

The liver extracts were prepared by freezing the weighed tissue in a cooled mortar; the tissue was thoroughly ground with a little sand and 0·067 M sodium phosphate buffer, pH 6·64, was added; the volume of buffer added varied with the amount and activity of the material available. The extracts were centrifuged for 30 min. at 2150 *g* at 0°.

It is to be noted that the pH of the phosphate buffer differed from that used in previous work from this laboratory. This change from pH 7·4 to 6·64 was adopted in order to reduce the CO_2 retention. Preliminary experiments, carried out using L-cysteine sulphinic acid, showed that the enzymic activity was nearly independent of pH.

The plan of the manometric experiments was similar to that used by Sloane Stanley (1949). The total reaction volume was 2·7 ml.; the initial substrate concentration was $1{\cdot}48 \times 10^{-2}$ M. Where stated, 10 μg. of pyridoxal phosphate were added to each flask. The gas phase was N_2 and the temperature was 37·5°. The enzymic activity was expressed as q_{CO_2}, i.e. as μl. CO_2 formed/mg. fresh wt. of tissue/hr., and was calculated from the initial rate of the decarboxylation.

RESULTS

Distribution of activity in normal liver extracts. It was found that liver extract from several animal species could decarboxylate both L-cysteic and L-cysteine sulphinic acids. The results of these experiments are summarized in Table 1. In all these experiments L-cysteine sulphinic acid was decarboxylated at a much faster rate than L-cysteic acid.

A typical experiment with dog liver is shown in Fig. 1. In this experiment the CO_2 formed from L-cysteine sulphinic acid was less than the theoretical amount. This was frequently observed. The cause of the deficit was not investigated, but it must be borne in mind that mammalian liver contains another enzyme, desulphinase, which acts upon L-cysteine sulphinic acid, giving alanine and sulphur dioxide (Fromageot, 1951).

Table 1. *Decarboxylation of* L-*cysteic and* L-*cysteine sulphinic acids by extracts of livers of different species*

Initial substrate concn., $1{\cdot}48 \times 10^{-3}$M. Gas phase, N_2; temp., 37·5°. The q_{CO_2} is defined as μl. CO_2 formed/mg. fresh wt. of tissue/hr. Sex indicated by M. or F. in brackets.

Species	No. of observations	L-Cysteic acid (q_{CO_2})	L-Cysteine sulphinic acid (q_{CO_2})	$\frac{q_{CO_2}\text{ L-Cysteic acid}}{q_{CO_2}\text{ L-Cysteine sulphinic acid}}$
Dog	1	0·18	1·24	0·15
Rat (M.)	5	0·12	1·01	0·12
Rat (F.)	6	0·10	0·76	0·13
Mouse (M.)	1	—	0·40	—
Guinea pig (F.)	1	0·07	0·52	0·14
Rabbit (M.)	1	0·02	0·04	0·50
Cat	4	0	0	—

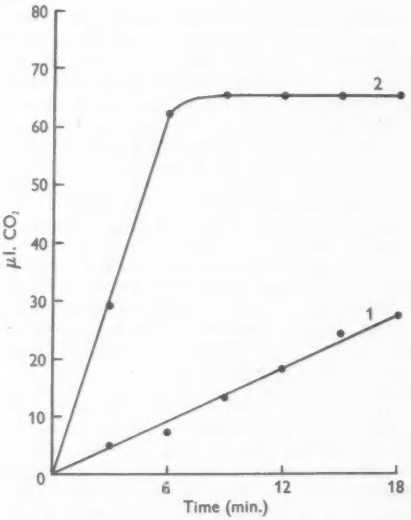

Fig. 1. Decarboxylation of L-cysteic and L-cysteine sulphinic acids by an extract of dog liver. Each manometer flask contained extract derived from 500 mg. of fresh tissue. Curve 1, $1{\cdot}48 \times 10^{-3}$M L-cysteic acid; curve 2, $1{\cdot}48 \times 10^{-3}$M L-cysteine sulphinic acid. Gas phase: N_2. Temp., 37·5°.

Table 2. *Rate of decarboxylation of* L-*cysteine sulphinic acid in male and female rat-liver extracts*

Experimental details as in Table 1.

	q_{CO_2}	
	Males	Females
	1·08	0·72
	1·02	0·74
	1·08	0·75
	0·93	0·88
	0·94	0·81
	—	0·66
Mean	1·01	0·76
S.E.M. (±)	0·033	0·031

Table 1 also shows that dog liver, which is known to have the highest activity with L-cysteic acid as substrate, was also highly active with L-cysteine sulphinic acid. In the cat, where no L-cysteic acid decarboxylase could be demonstrated, no CO_2 was formed from L-cysteine sulphinic acid. The results with rat liver confirm Sloane Stanley's (1949) observation that the mean L-cysteic acid decarboxylase activity is higher in males than in females. The results in Table 2 show that the same is true for L-cysteine sulphinic acid.

Competition experiments. The great difference in the rate of decarboxylation of the two amino acids made the study of substrate competition difficult, but an additive formation of CO_2 never occurred when the two acids were added together. In an experiment with a rat-liver extract, in which 10 μg. of pyridoxal phosphate were added to each manometer flask, the amounts of CO_2 formed in the first 6 min. were: with $1{\cdot}85 \times 10^{-3}$M L-cysteic acid, 4 μl.; with $1{\cdot}85 \times 10^{-3}$M L-cysteine sulphinic acid, 58 μl.; with both (each $1{\cdot}85 \times 10^{-3}$M), 44 μl.

Observations on pyridoxine-deficient rats. The earlier observations that decarboxylase activity for both L-cysteic and L-cysteine sulphinic acids disappear from the liver of rats fed on a pyridoxine-deficient diet were confirmed. The results of these experiments are summarized in Table 3.

It can be seen that the ability to decarboxylate the two amino acids was lost very rapidly; at the end of a fortnight no activity remained. Most of the activity towards both substrates was lost in a few days.

The action of synthetic co-decarboxylase (pyridoxal 5-phosphate) on liver extracts. It was noted that the addition of synthetic pyridoxal phosphate to liver extracts from normal rats sometimes increased the decarboxylase activity. This is shown in Table 3; the increases in activity for the group of seven normal rats were 0, 3, 25, 17, 20, 39 and 0%, respectively, with L-cysteine sulphinic acid as substrate.

This observation, together with the loss of enzymic activity in pyridoxine deficiency, sug-

Table 3. *Decarboxylase activity in normal and vitamin B_6-deficient rat-liver extracts*

Where pyridoxal phosphate was added, 10 μg. were used in a total volume of 2·7 ml. Other experimental details as in Table 1.

		Without added pyridoxal phosphate		With pyridoxal phosphate added	
Sex	Days on vitamin B_6-deficient diet	L-Cysteic acid (q_{CO_2})	L-Cysteine sulphinic acid (q_{CO_2})	L-Cysteic acid (q_{CO_2})	L-Cysteine sulphinic acid (q_{CO_2})
F.	0	0·11	0·75	0·11	0·75
M.	0	0·12	1·02	0·12	1·05
F.	0	0·14	0·72	0·14	0·90
M.	0	0·14	1·08	0·16	1·26
F.	0	0·06	0·74	0·08	0·89
F.	0	0·12	0·88	0·11	1·22
M.	0	0·13	0·94	0·12	0·94
F.	2	0·05	0·28	0·06	0·53
M.	3	0·08	0·60	0·12	1·05
F.	3	0·02	0·16	0·05	0·31
F.	3	0·02	0·24	0·09	0·72
F.	4	0	0·07	0·05	0·23
F.	4	0	0·06	0·03	0·18
F.	4	0	0·04	0·03	0·095
M.	11	0	0·01	0	0·08
M.	14	0	0	0	0

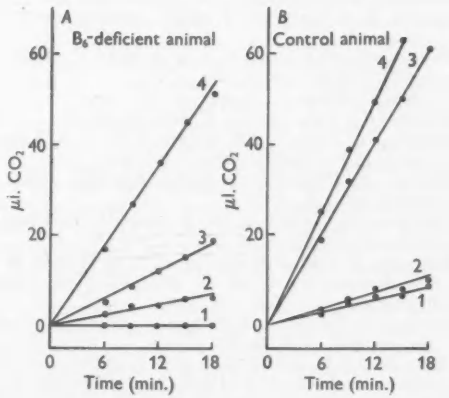

Fig. 2. Decarboxylation of L-cysteic and L-cysteine sulphinic acids by rat-liver extracts. *A*, Female rat deficient in pyridoxine for 3 days; *B*, female rat litter mate on supplemented diet. Each manometer flask contained extract derived from 250 mg. of fresh tissue. Curve 1, $1{\cdot}48 \times 10^{-3}$M L-cysteic acid; curve 2, $1{\cdot}48 \times 10^{-3}$M L-cysteic acid plus 10 μg. pyridoxal phosphate; curve 3, $1{\cdot}48 \times 10^{-3}$M L-cysteine sulphinic acid; curve 4, $1{\cdot}48 \times 10^{-3}$M L-cysteine sulphinic acid plus 10 μg. pyridoxal phosphate. Gas phase: N_2. Temp., 37·5°.

gested that the decarboxylation was catalysed by an enzyme containing pyridoxal phosphate. Experiments were therefore carried out with liver extracts from pyridoxine-deficient rats in which pyridoxal phosphate was added in order to find out whether the enzymic activity could be re-established.

Reactivations with pyridoxal phosphate could not be obtained with liver extracts from animals which had been on the deficient diet for 2 weeks or more, and only very slight effects were seen after the first week. However, an effect of pyridoxal phosphate added *in vitro* was observed in a number of experiments in which the rats were killed during the first week of deficiency, i.e. on the second to the fourth day. At this time the activity towards both amino acids was found to be markedly reduced. Fig. 2 shows one of these experiments in which the rates of decarboxylation of both amino acids were measured with and without added pyridoxal phosphate; the experimental animal, a female, had been on the pyridoxine-deficient diet for 3 days. The rates of decarboxylation of the two acids by the pyridoxine-supplemented control animal, a female litter mate, are also shown.

DISCUSSION

The experiments described give strong support to the suggestion of Awapara & Wingo (1953) that the enzyme which acts on L-cysteic acid is also responsible for the decarboxylation of L-cysteine sulphinic acid. In all species examined the rate of decarboxylation of the sulphinic acid is greater than that of cysteic acid. This makes it likely that L-cysteine sulphinic acid is the chief substrate for the enzyme in the living organism. The observations also explain why L-cysteic acid can act as a precursor of taurine (Virtue & Doster-Virtue, 1939).

One fact concerning taurine synthesis which remains unexplained is the absence of the decarboxylase in the liver of animals like the cat, which

forms taurocholic acid. A number of explanations are possible. Either there exists a different pathway which does not require the presence of L-cysteine sulphinic acid, or the decarboxylase occurs in an organ other than the liver, or these animals depend upon the taurine taken in with the diet. At present there is no means of distinguishing between these possibilities. The problem of taurine formation has become of greater interest since it has now been established that free taurine occurs in the central nervous system (Roberts, Frankel & Harman, 1950).

The activating effect of pyridoxal phosphate on liver extracts from pyridoxine-deficient animals establishes the chemical nature of the coenzyme. Earlier attempts at reactivation had been unsuccessful; this can now be understood as it has been shown that reactivation is possible only during the first few days of deficiency. This result can best be interpreted by assuming that in the pyridoxine-deficient rat the depletion in apoenzyme follows the loss of coenzyme with only a brief time lag. It would seem that reactivation is possible only while some enzymic activity remains; the enzymic activity is rarely more than trebled.

Successful reactivation of the enzyme L-dopa decarboxylase of the pyridoxine-deficient rat liver has previously been reported (Blaschko, 1950); this enzyme disappears at a slower rate than L-cysteic acid decarboxylase and reactivation can be more easily demonstrated. It thus appears that in the deficient animal there exists a time sequence with which the different pyridoxal phosphate enzymes disappear. The work here reported shows that there are three different sequences of this kind. The first is the disappearance of coenzyme bound to apoenzyme; the second, the disappearance of the enzyme protein; the third sequence is the disappearance of co-decarboxylase in the liver. It has already been shown that with both decarboxylases the loss of enzymic activity is either complete or almost complete when large amounts of coenzyme are still present (Blaschko *et al.* 1948).

SUMMARY

1. The decarboxylation of L-cysteic and L-cysteine sulphinic acids by extracts of mammalian liver has been studied; the evidence reported suggests that one enzyme is responsible for the decarboxylation of both these amino acids.

2. Enzymic activity is rapidly lost in pyridoxine-deficient rats; reactivation by the addition of pyridoxal 5-phosphate *in vitro* has been obtained in the early stages of the deficiency; this observation establishes pyridoxal phosphate as the coenzyme of the decarboxylase.

3. The biosynthesis of taurine is discussed in the light of these findings.

I wish to thank Professor J. H. Burn, F.R.S., for the privilege of working in his laboratory and Dr H. Blaschko for his guidance throughout. The work has been done during the tenure of a fellowship given by Messrs Parke, Davis and Co., to whom I wish to express my thanks.

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Comirin: Isolation and Properties

By W. G. C. FORSYTH

Colonial Microbiological Research Institute, Trinidad, British West Indies

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Comirin, a new fungistatic antibiotic, is produced by a bacterium found accidentally on a medium used for the cultivation of a species of *Aspergillus*. The bacterium was identified as a new species, related to *Pseudomonas fluorescens*, and given the name of *P. antimycetica* n.sp. (Thaysen & Thaysen, 1953).

The characteristics of the bacterium and the culture conditions for comirin production will be reported separately (Thaysen & Thaysen, in preparation). Seven days' growth in peptone (0·5 %)–sucrose (0·5 %) medium was generally used.

In the following pages, the method of assay elaborated for following the efficiency of the ex-

traction procedure, the method of isolation, and some chemical and physical properties of comirin are described.

ISOLATION

Assay. Activity is measured by an 'agar-cup' plate diffusion method. The test organism is a strain of *Aspergillus flavus* (Hankey Culture Collection, F9) added as a standard suspension of spores to a medium containing glucose (0·5%), marmite (0·25%), peptone (0·5%), agar (2·0%).

Plates containing fifty 8 mm. holes are used to compare five solutions, each solution being added to ten holes at random. The solutions are heated before testing (to 90° from room temperature within 10 min.) in a water bath to pasteurize and also to disperse the sparingly soluble comirin. Four drops are added to each hole from a standard dropper. The plates are incubated at 30° for 24 hr. The ring sizes are measured to the nearest 0·5 mm. The main error is in the measurement. This error was more or less constant and could not be reduced by magnifying the zones since it was a function of the irregularity of the inhibition edge of the zones. The average standard deviation between replicates under these conditions is $\pm 0{\cdot}7$ mm. and between means is $\pm 0{\cdot}3$ mm. when measuring zones of 18–25 mm. diameter. This corresponds to an accuracy within the fiducial limits at the 5% level of probability of 75–125%. Between 18 and 25 mm. the diameter of the zone of inhibition is directly proportional to the logarithm of the concentration.

Preliminary experiments. It is found that when active culture solutions are centrifuged, only a fraction of the activity remains in the cleared solutions. Over a pH range of 3–9 there is no significant variation in the amount of activity retained by the bacteria (41 ± 4%). It is immaterial whether the solutions are pasteurized before or after centrifuging, i.e. either comirin is sparingly soluble in the media in this pH range, or it is retained adsorbed on inactive cell debris. The adsorbed or water-insoluble, active fraction can be brought into solution with aqueous alcohols, acetone, dioxane or pyridine.

Comirin cannot be extracted from active media with light petroleum, benzene, ether, chloroform, amyl acetate or ethyl acetate at any pH. In fact, shaking with these solvents partly precipitates the activity at the interface. Comirin can be extracted by *n*-butanol.

The activity is precipitated by heavy metal acetates, picric acid, tannic acid, phosphotungstic acid, and other general protein precipitants. It is also precipitated on part saturation with $(NH_4)_2SO_4$, Na_2SO_4 or $MgSO_4$ or with NaCl. A method of isolation via initial $(NH_4)_2SO_4$ precipitation was worked out but discarded, since it was much more laborious and considerably less effective than the simple method finally adopted. It will not therefore be reported.

The activity is completely adsorbed by activated charcoal or alumina. It cannot, however, be eluted from them by buffers, alcohols, or acid alcohols, and is only recovered in part on elution with aqueous pyridine. A striking property of comirin is its adsorption by cellulose. On simply filtering 10 ml. of a purified aqueous solution containing 0·4 mg. of comirin through a 10 cm. Whatman no. 1 filter paper in a conical filter, over 90% is adsorbed. This can be eluted with aqueous alcohols or pyridine.

Extraction with n-butanol. Although it is possible to concentrate comirin either by initial precipitation or by adsorption, the fact that the activity is partly adsorbed by the bacterial debris limits the usefulness of these methods. Extraction with an immiscible solvent would seem the most likely approach. Early efforts were expended in extracting the activity from salt-saturated media with ethanol or *n*-propanol, but this was abandoned in favour of direct butanol extraction.

The active culture medium has a pH *ca.* 7, and no advantage is gained by altering the pH. Extremes of pH increase the losses during extraction. Using a 1/4 volume of butanol does not give complete extraction. Although practically all the comirin is removed from the aqueous layer, it is apparently not very soluble in water-saturated butanol and much remains at the interface. Using continuous, or batch extractions on the countercurrent principle, better extraction could of course be obtained. It was found, however, that by raising the temperature of the butanol–water gel, a much larger proportion of comirin passed into the butanol. In this way very high extraction is obtained in a single step, readily adaptable to working with large volumes. There is an optimum temperature at 70°, because at higher temperatures a gel-forming material passes into the butanol and decreases the recoveries at the later stages.

Recovery from butanol. (1) Concentration at low pressure. It is found that, when the butanol extract is concentrated at low pressure at room temperature to small volume, the active material precipitates from the concentrate as a white powder. Generally about 70% of the activity is recovered. (2) Displacement with immiscible solvents. An equal volume of solvent is added to the water-saturated butanol, giving an organic and aqueous layer. The activity can be recovered from the interfacial precipitate in the following yields: light petroleum (72%); ether (50%); amyl acetate (86%). (3) Dehydration with salt (cf. Fevold, Dimick & Klose, 1948). The butanol is shaken with 4% (w/v) of NaCl and left overnight. An inactive saline layer separates off. The butanol layer contains 2% of the activity and 91% can be recovered from the interfacial precipitate. This method is routinely used.

Further purification. Aqueous pyridine (50%, v/v) is found to be by far the best extractant of the activity from the saline precipitate. The activity can then be recovered in high yield from the aqueous pyridine by addition of amyl acetate or ethyl acetate and once again recovering the interfacial precipitate.

Final procedure. The following is a typical extraction flow sheet. The figures in brackets are the amounts of comirin in each fraction as determined by assay:

The active culture in 90 l. lots ($\equiv$2 g. comirin) is stirred vigorously for 3 hr. with 22·5 l. *n*-butanol. After standing overnight, the aqueous layer (130 mg. comirin) is siphoned off and discarded. The gel remaining is heated to 70° for 1 hr. and separated on a Sharples centrifugal separator at 50–70°. The water from the gel (120 mg. comirin) may be added to the next batch if desired. The interfacial precipitate (150 mg. comirin) is discarded.

The butanol extract (1·6 g. comirin, 80%) with 4% (w/v) NaCl added, is stirred for 2 hr., and allowed to settle. The butanol layer is siphoned off and the interfacial precipitate (1·43 g. comirin) separated from the aqueous solution by centrifuging. The precipitate is extracted, by shaking and centrifuging, with three successive lots (500, 300, 200 ml.) of 50% (v/v) pyridine (freshly redistilled)–water. The combined pyridine extracts are then shaken with 4 vol. of amyl acetate and the organic layer discarded. The white

gelatinous interfacial precipitate is collected, washed with a small volume of absolute ethanol, then acetone, and dried *in vacuo*. The comirin is thus obtained as a white amorphous powder. Yield; 1·28 g. (64%).

1 mg. of this preparation in 37 ml. water gave a clearing zone of 23 mm. in the standard assay. By the above method it is readily obtained in 60–70% yield.

Evidence of homogeneity

Paper chromatography. Comirin was chromatographed by a simple ascending-solvent one-dimensional strip technique and detected by laying the developed air-dried strips on agar media seeded with the *Aspergillus flavus* used as test organism (Forsyth, 1953).

Comirin moves as a single discrete spot in water-saturated amyl alcohol (R_F, 0·05); water-saturated *n*-butanol (R_F, 0·16); *n*-butanol (2 parts)–pyridine (1 part)–water (4 parts), (R_F, 0·42); aqueous ammonia sp.gr. 0·880 (R_F, 0·75); water-saturated phenol (R_F, 0·81); 60% (v/v) aqueous dioxane (R_F, 0·88); 25% (v/v) monoethylamine (R_F, 0·92); 60% (v/v) pyridine–water (R_F, 0·96). It remains on the starting line with water. In no case is more than one spot obtained, even with two-dimensional descending solvent systems using pairs of the above.

Countercurrent distribution. The distribution of the activity during a ten-tube diagonal countercurrent distribution analysis (Bush & Densen, 1948), using 30 mg. of comirin and a mixture of *n*-butanol (1 part) and water containing 10% pyridine (5 parts) is shown in Fig. 1.

Other evidence. It would appear that the biological effect is due to one substance. The following experiments suggest that no inactive impurities are present: (*a*) Dissolving in aqueous pyridine and reprecipitating at the interface does not increase the activity. (*b*) Fractional precipitation from pyridine or acetic acid solution with ether, ethyl acetate or amyl acetate, does not give any fractions of increased activity. (*c*) Comirin is much more soluble in boiling water than in cold. Fractionation by dissolving in boiling water and cooling does not lead to any increase in activity. (*d*) Precipitates obtained by cooling hot saturated aqueous pyridine solutions do not show an increase in activity. (*e*) Comirin was recovered from fractions 8, 9 and 10 of the above countercurrent analysis. The material isolated has the same activity as the original. (*f*) Comirin, chromatographed in a column of packed cellulose pulp with the top layer of a mixture of *n*-butanol–pyridine–water (2:1:4, v/v), moves as a single band. On recovery from the active fraction of the eluate, the recovered material is of the usual activity.

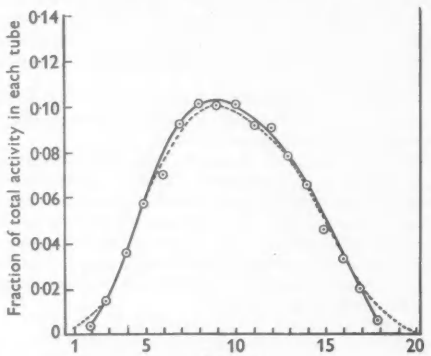

Fig. 1. Countercurrent distribution of comirin (30 mg.) in *n*-butanol–10% (v/v) aqueous pyridine (1:5, v/v). Ten-tube diagonal system. 90 ml. solvent/tube. - - - - Theoretical. $p = 0{\cdot}52$, ⊙—⊙ experimental (for definition of p see Bush & Densen, 1948).

It would appear that, pending results from other physical methods (ultracentrifuge, electrophoresis, etc.), the material may be considered reasonably homogeneous.

Purified preparations of comirin are haemolytic. That this is a property of the antibiotic itself, and not due to a haemolytic contaminant can be readily shown. It is possible to detect haemolytic substances on paper chromatograms by placing the developed chromatograms on saline agar plates containing washed blood cells. Zones of lysis of the blood cells are formed under the influence of these separated haemolytic substances. The identity or otherwise of the antibiotic and haemolytic substances can then be found by covering the blood cell plates with a thin layer of nutrient agar containing a test organism and incubating. If the two activities are due to a single substance, then the centres of the two zones will coincide and the shape, but not necessarily the area, of the two zones will be identical. When comirin was tested in this manner using eight different solvent systems, in every case the zones of inhibition and lysis coincided. Moreover, the diameters of the two zones were practically equal.

PROPERTIES

Solubility. The solubility of comirin at 25° in various solvents is shown in Table 1.

Stability of comirin. Comirin (13 mg.) was dissolved in water (500 ml.) and divided into 9 × 50 ml. lots. Each lot was made up to 100 ml. with water and dilute HCl or NaOH to give the desired pH in the range 3–9 (Table 2). The solutions were then assayed after pasteurization (by heating to 90° from room temperature within 10 min. in a water bath) and after further heating (100° for 30 min.) under reflux.

While there is a loss at alkaline pH values on continued heating, there is also a highly significant apparent increase at pH ⩽ 4 even with the heating necessary for assay only.

Table 1. *Solubility of comirin at* 25°

	mg./100 ml.
Water	4
50% Aqueous ethanol	12
50% Aqueous acetone	18
25% Monoethylamine	140
Pyridine	170
50% Aqueous pyridine	300
Glacial acetic acid	320

Insoluble in: anhydrous alcohols, acetone, dioxan, ether, ethyl acetate, amyl acetate, chloroform, benzene, light petroleum.

Table 2. *Stability of comirin*

A comirin solution of known concentration (by weighing) adjusted to the pH values shown and assayed before and after heating (30 min. at 100°). Results as percentage original. For conditions of assay see text. A significant difference at the 5% level = +25%. Italic type, significant; clarendon type, highly significant difference from original.

pH	9·0	8·0	7·5	7·0	6·5	6·0	5·0	4·0	3·0
Assay (includes pasteurization)	97	97	101	97	108	108	93	*144*	**176**
Assay after further heating (30 min. 100°)	**35**	*64*	101	108	104	104	97	**176**	**206**

Table 3. *Dispersion and aggregation*

A comirin solution of known concentration by weighing: *A*, pasteurized at pH 7; *B*, heated 15 min. at 100° at pH 3; *C*, *B* readjusted to pH 7. The solutions *A*, *B* and *C* then allowed to stand 24 hr. at 25°. Results as percentage original. (H.S., highly significant; N.S., not significant).

	A	*B*	*C*
pH ...	7	3	3–7
Assay after treatment	100	178	180
After 24 hr. standing	85	115	80
After re-pasteurizing	88	185	85
Loss on standing	N.S.	H.S.	H.S.
Gain on re-pasteurizing	N.S.	H.S.	N.S.

A preparation of comirin shown to be homogeneous with regard to chromatography (4 mg.), was pasteurized with 100 ml. water. The pH was then adjusted to 3·0 and the solution boiled for 15 min. The solution was then adjusted back to pH 7. After 24 hr. the solutions were again tested before and after pasteurizing. The results are shown in Table 3.

Dialysis. When an active, aqueous solution of the antibiotic is suspended in a cellophan sac, no dialysis through the cellophan can be detected. However, when the organism is streaked on sterile cellophan, placed on glucose–marmite–peptone–agar containing fungal spores and incubated, a very slight zone of inhibition is observed directly below the streak. This zone was only the width of the streak (*ca.* 2 mm.), whereas spraying fungal spores over the top of the cellophan showed that an inhibition zone of *ca.* 15 mm. had been produced. The antibiotic must be very sparingly dialysable in aqueous solution through cellophan. Using a very active solution, only 0·2% dialysed in 24 hr. When, however, solutions which had been pasteurized at acid pH were dialysed, the following results were obtained after 24 hr. as percentage dialysed; (1) pasteurized at pH 7, 0·3%; (2) at pH 3, 3·1%: (3) pasteurized at pH 3 and adjusted back to pH 7, 3·0%.

Chemical analysis and tests. (*a*) Comirin contains no sulphur, halogens, phosphorus or ash. Air-dried samples contain 5–7% moisture. The following analyses were obtained on a moisture-free basis: C, 49·08, 48·76, 48·86; H, 6·70, 6·86, 7·00; N (Dumas), 11·88, 12·18, 11·73; N (Kjeldahl), 12·09, 11·91, 11·88%. The mean N value, 11·9%, is too low for a simple peptide. The antibiotic melts with decomposition over the range 230–235° depending on the rate of heating. All attempts at crystallization have been unsuccessful. (*b*) Comirin gives positive Millon-Cole, Pauly, Sakaguchi and xanthoproteic reactions. A positive biuret reaction is obtained but a negative ninhydrin reaction. The activity is unaffected on boiling with ninhydrin. Glyoxylic acid and Molisch tests were also negative. The activity is not destroyed by pepsin, papain or trypsin. (*c*) Comirin (6 mg.) was hydrolysed with 0·6 ml. 6N-HCl for 18 hr. in a sealed tube at 110°. An average of 90% of the total nitrogen is liberated as α-amino acid nitrogen (ninhydrin–carbon dioxide volumetric method). The original material contains no free α-amino acid N as measured by this method. The antibiotic is soluble in 6N-HCl to give a clear, colourless solution, but after 1 hr. heating, a copious precipitate separates out on cooling. At this stage ether-soluble material (*ca.* 20%) has been liberated. On further heating the hydrolysate darkens and 6% of dark 'humin' forms. An equal volume of water was added and this precipitate was centrifuged off. The supernatant was evaporated several times to dryness *in vacuo* with addition of water to remove HCl. The residue was taken up in 0·3 ml. of water and subjected to paper chromatography (Consden, Gordon & Martin, 1944).

The apparatus used for two-dimensional chromatography was essentially that of Dent (1948). However, the chromatogram was run in one direction with phenol in the presence of NH_3 and NaCN, and in the other with the butanol–acetic acid mixture of Partridge (1948). Whatman no. 1 paper was used throughout. The chromatograms were sprayed with ninhydrin (0·1%, w/v) in butanol and the strength of the spots detected was estimated visually from their area and colour intensity.

Identification of ninhydrin-reacting substances. Most of the amino acids were readily identifiable from the pattern of the spots, the chromatograms being compared with those of synthetic mixtures of pure amino acids run under identical conditions. In many cases identification was checked by adding pure amino acids to the hydrolysates. It was shown by the $CuCO_3$ technique of Crumpler & Dent (1949), which distinguishes α-amino acids from other

ninhydrin-reacting substances, that one spot present —no. 13—was not due to an α-amino acid. The nature of this substance is not known.

Leucines (no. 1). It was readily shown by one-dimensional chromatography with *tert.*-amyl alcohol (Work, 1949) that both leucine and isoleucine were present. Methionine could not be detected by ninhydrin, or the H_2O_2 technique (Dent, 1947), even when the chromatograms were overloaded with the other amino acids.

Tyrosine (no. 3). Although the original hydrolysate gave quite a strong Millon-Cole reaction, most of the colour producing substances were in the black insoluble precipitate formed (cf. Gordon, Martin & Synge, 1943) and only a trace of tyrosine was detected in the solution after centrifuging. This was barely visible on the two-dimensional chromatograms, but its presence could be shown by a one-dimensional chromatogram run in butanol–acetic acid (Partridge, 1948) and sprayed with diazotized sulphanilic acid followed by Na_2CO_3 solution (Pauly, 1904). The absence of histidine was also shown with this reagent.

The basic amino acids (nos. 10, 11, and 12). These three substances were shown to be basic, since they were retarded when phenol was used with an acetic acid instead of an ammonia atmosphere. Spots nos. 10 and 11 were readily identified by conventional methods as arginine and lysine. Spot no. 12 was separable from added ornithine. Other possibilities are α-γ-diaminobutyric acid which has been found in the polymyxin antibiotics (Jones, 1949) and α-ε-diaminopimelic acid which has been found in *Corynebacterium diphtheriae* (Work, 1950) and soils (Bremner, 1950). α-ε-Diaminopimelic acid, however, moves in a different position, appearing under glutamic acid (Bremner, 1950). α-γ-Diaminobutyric acid synthesized from glutamic acid could not be distinguished from the unknown on the above chromatogram. The two spots coincided when the chromatograms were run both in ammonia and in acetic acid atmospheres.

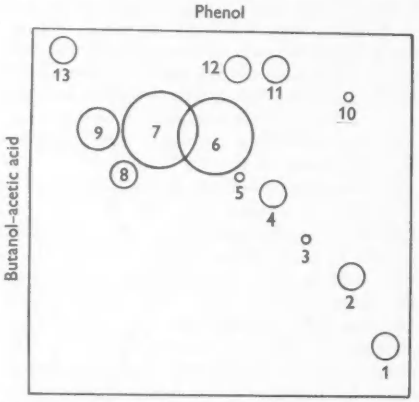

Fig. 2. Diagram of two-way chromatogram of the amino acids in a comirin hydrolysate. Solvents: water-saturated phenol; *n*-butanol–acetic acid–water (4:1:5, v/v). First run in phenol from left to right, then in butanol–acetic acid downwards. Intensity of spots approximately represented by area of circles. (Provisional identification: 1, leucines; 2, valine; 3, tyrosine; 4, alanine; 5, threonine; 6, glycine; 7, serine; 8, glutamic acid; 9, aspartic acid; 10, arginine; 11, lysine; 12, α-γ-diaminobutyric acid; 13, unknown).

'Unknown' (no. 13). This spot gave an atypical ninhydrin reaction. A brownish yellow spot first appeared which slowly changed to the normal purple. Although qualitative tests on the original antibiotic for sulphur were consistently negative, and methionine was absent, tests were made for cystine or sulphur-containing breakdown products. The H_2O_2 technique failed to reveal cystine, although cystine was readily detected when added to the hydrolysate. Also, spraying with potassium iodoplatinate (Winegard, Toennies & Block, 1948) failed to detect any sulphur-containing amino acids. The sensitive spot test for cystine described by Dent & Rose (1949) was also negative. The substance did not form a salt on treatment of the paper with $CuCO_3$, and is not therefore an α-amino acid.

Dinitrophenyl (DNP) derivative of the antibiotic. Comirin readily reacts with 2:4-dinitrofluorobenzene (DNFB) to form a DNP derivative (Sanger, 1945). The antibiotic (0·1 g.) was suspended with 0·1 g. $NaHCO_3$ in 1 ml. of water, 0·1 ml. DNFB in 2 ml. ethanol was added and the mixture shaken for 2 hr. It was then extracted successively with water, ethanol and ether leaving a bright yellow solid practically insoluble in water and ethanol, but readily soluble in pyridine. That the reaction was complete could be shown by paper chromatography with 60% (v/v) acetone. Under these conditions comirin has an R_F of about 0·8, whereas both the colour and activity (the DNP derivative is active) remain on the starting line.

The DNP comirin was hydrolysed for 18 hr. with 5N-HCl and the hydrolysate then extracted with ether. The ether extract and the water layer were prepared as usual (Sanger, 1945) and subjected to paper chromatography. All the colour was confined to the aqueous layer. The aqueous layer gave two water soluble spots, major component (R_F, butanol–acetic acid, 0·64) minor component (R_F, 0·73). They are found close to the leucines in the two-way chromatogram.

Substrate. To determine whether there was any correlation between the amino acid composition of the comirin produced and the protein substrate, 20 amino acids were tested individually as the sole source of nitrogen. The medium used was as follows:

Table 4. *Production of comirin on amino acids as sole source of nitrogen*

For conditions see text.

Inoculum: Slope ...	A		B		C		D			
Inoculum: Colony ...	1	2	1	2	1	2	1	2		
Nitrogen substrate	Inhibition zones (mm.)								Active cultures (%)	Mean clearing zones
Peptone	22	23	22	20	19	19	17	18	100	20·0
Ammonium citrate	21	20	17	17	18	19	17	16	100	18·1
Ammonium phosphate	18	19	19	18	19	19	12	14	100	17·2
L-Histidine	22	20	17	16	19	18	20	19	100	18·8
L-Arginine	18	21	20	17	19	17	21	—	88	17·6
L-Asparagine	22	19	19	20	15	—	16	—	75	15·9
DL-Alanine	22	9	9	12	15	—	12	—	75	11·9
DL-Glutamic acid	20	—	—	18	—	—	—	13	38	—
DL-Aspartic acid	20	—	—	—	—	—	—	—	12	—
L-Lysine	—	—	—	—	9	17	15	17	50	—
DL-Tryptophan	—	—	—	—	—	19	—	16	25	—

Glucose, 0·5 %; K_2HPO_4, 0·1 %; KH_2PO_4, 0·1 %; $MgSO_4$, 0·04 %; $FeSO_4$, 0·002 %. The nitrogen compounds were added in the proportion of 75 mg. N per 100 ml. and the pH adjusted to 6·5.

Loopfuls from four different slopes of *Ps. antimycetica* were plated out at high dilutions and two colonies picked at random from a suitable plate from each slope. These were suspended in eight 10 ml. lots of saline and one saline tube was used to inoculate each set of media in 10 ml. lots in test tubes. One drop was used for each inoculum. In this way the effect of strain variation (Thaysen & Thaysen, in preparation) could be overcome. After incubation for 7 days at 30° growth was noted and the activity determined. In Table 4 are listed the nitrogen compounds which supported good growth and gave some activity.

It would appear that the organism is able to utilize under these conditions, only those amino acids which either play a major part in protein synthesis (alanine, glutamic acid and aspartic acid), or contain a nitrogen grouping in addition to the α-amino N (histidine, asparagine, arginine, lysine and tryptophan).

DISCUSSION

A simple method for the isolation of comirin in an apparently homogeneous form has been developed, based on its rather peculiar solubility properties. Comirin is readily isolated by repeated precipitation at the interface between organic and aqueous solutions. Throughout this investigation the technique of paper chromatography has proved extremely useful as a guide to isolation procedures.

Comirin on hydrolysis gives 14 ninhydrin positive substances of which 12 are readily identified as common amino acids. An uncommon basic amino acid is present which is probably α-γ-diaminobutyric acid. The fourteenth ninhydrin-reacting substance is not an α-amino acid. Glycine, serine, and to a lesser extent, aspartic acid, make up the bulk of the peptide moiety. There is no simple relationship between the amino acids useful as a substrate and the constituents of the peptide. The different strains vary greatly (Table 4) in their ability to produce comirin from various amino acids.

Comirin solutions when heated at pH ≤ 4 show a large increase in apparent activity. On standing the activity returns to normal. There is no evidence of a chemical dissociation. On paper chromatography the same R_F values are obtained whether the material has been 'activated' at pH 4 or not. Comirin probably exists in aqueous solution as a colloidal suspension, which is dispersed on heating at acid pH values and reaggregates on standing. It is very sparingly dialysable through cellophan.

The only substances with which comirin might be confused are the various antifungal antibiotics from *Bacillus subtilis*, and *B. polymyxa*. However, the solubility, lack of activity against bacteria, and the amino acid composition of comirin, clearly differentiate it from these other substances. Comirin is readily distinguished from other antifungal agents produced by fungi and actinomycetes by paper chromatography (Forsyth, 1953).

No non-pigmented antifungal substance has previously been isolated from a non-spore-forming bacterium, although the closely related species *Ps. fluorescens* has been reported to have antifungal activity, which however can also be readily distinguished from comirin by paper chromatography.

SUMMARY

1. A method of isolating comirin from active culture media, in what is believed to be a homogeneous form, in 60–70 % yield is described.

2. The homogeneity was checked by chromatography and countercurrent analysis.

3. Comirin is a peptide containing an ether-soluble moiety.

4. The antibiotic shows a striking apparent increase in activity on heating in acid solution.

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A New Method for the Determination of the Amidase Activity of Trypsin: Kinetics of the Hydrolysis of Benzoyl-L-Arginineamide

By S. A. BERNHARD*
Department of Colloid Science, University of Cambridge

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The determination of the amidase activity of trypsin is complicated by the appreciable rate of self-digestion of the enzyme under optimum conditions for the hydrolysis. In order to obtain significant results it was necessary to find a kinetic procedure by which the hydrolysis could be followed rapidly relative to the rate of inactivation of the enzyme. In the course of tests of the formol titration method of Iselin & Niemann (1950) it was observed that the esterase activity of trypsin was unaffected by formaldehyde. Esterase activity was followed by the potentiometric titration of Schwert, Neurath, Kaufman & Snoke (1948) and of Schwert & Eisenberg (1949) with benzoyl L-arginine ethyl ester (BAEE) as substrate. The fact that the activity of trypsin is unaffected by formaldehyde (as determined by esterase activity) was made the basis of a potentiometric method for following the amidase activity. At the pH-activity optimum (pH 7·5–8·0), in the presence of formaldehyde the trypsin-catalysed hydrolysis of amides may be represented by the following equations:

$$RCONH_2 + H_2O \rightarrow RCO_2^- + NH_4^+,$$

$$4NH_4^+ + 6CH_2O \rightarrow (CH_2)_6N_4 + 4H_3O^+ + 2H_2O.$$

Thus, in the case of trypsin, the hydrolysis of amides can be followed directly by measuring the amount of oxonium ions liberated in the presence of formaldehyde. This method has been successfully carried out employing benzoyl-L-arginineamide (BAA) as substrate.

* Present address: Naval Medical Research Institute, Bethesda, Md., U.S.A.

MATERIALS AND METHODS

Substrates. Benzoyl-L-arginineamide and ethyl ester were prepared from L-arginine hydrochloride (British Drug Houses Ltd., London) as described previously (Bergmann, Fruton & Pollok, 1939). Acetyl-L-phenylalanine (I) was prepared, according to the method of Huang & Niemann (1951), from L-phenylalanine (supplied by Roche Products Ltd., Welwyn, Herts). The ethyl ester was prepared by esterification of I in HCl-saturated ethanol at room temperature. After 2 days the reaction mixture was partially evaporated under vacuum and precipitated with water. The crude ester was recrystallized twice from hot water (pH 7).

Enzymes. Trypsin: the trichloroacetic acid precipitate of Northrop, Kunitz & Herriott (1948) was suspended in water and exhaustively dialysed against 0·01 M-HCl until the protein all dissolved (about 3 days at 5°). Crystallization of this material resulted in no further increase in activity and hence the dialysed samples at pH 2 were stored and used in the rate experiments.

Chymotrypsin: chymotrypsin was prepared by Dr H. Gutfreund from the trypsin digest of a sample of chymotrypsinogen (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) by the method of Northrop *et al.* (1948). Before use a 0·1% solution was dialysed against 0·005 M ethylenediamine (pH 7·5) solution.

Method. The same procedure was followed in the measurement of all trypsin-catalysed rates. The reaction was carried out in a 20 ml. beaker immersed in a water bath maintained at 25·00±0·05°. In the beaker were placed 10 ml. of solution containing substrate, 0·1 M-Na_2HPO_4–KH_2PO_4 buffer (pH 7·6) and 0·2 M-NaCl. When the substrate was the amide 0·5 ml. of 40% (w/v) formaldehyde (neutralized to pH 7·5 with NaOH) was added in addition. This concentration of formaldehyde was found to be adequate (see Fig. 1). Approximately 0·1 N-NaOH, standardized in terms of μ equivalents/micrometer division, was added to the reaction mixture by means of a hypodermic syringe firmly connected to a micrometer screw. The needle of the syringe dipped into the reaction mixture. The pH was measured with a model 505 pH meter (Pye Instrument Co., Cambridge). At zero time, 0·2 ml. of enzyme solution was added, the pH adjusted to slightly over 7·60 and the time at which the needle crossed the 7·60 mark recorded. Small amounts of base were added immediately following the time readings. Maximum deflexions were about 0·05 pH unit (no change in activity between pH 7·5 and 8·0 could be noted). The mixture was stirred throughout by bubbling through it CO_2 free nitrogen at 25°, saturated with water. Chymotrypsin-catalysed rates were followed similarly, with the exception that a 0·005 M ethylenediamine buffer was used to follow the reaction at pH 7·90. Specific rates could be determined with a precision of ±0·2% (the mean deviation from least squares analysis) and accuracy of ±1% (the reproducibility of duplicate experiments).

Effect of formaldehyde on the trypsin- and chymotrypsin-catalysed rates of hydrolysis of esters

In the following trypsin experiments, the activity of the enzyme was determined.

(1) One ml. of 0·5% trypsin solution in 0·01 N-HCl was mixed with 1 ml. of 40% (w/v) formaldehyde and allowed to stand at 25° for 2 min. The mixture was then diluted to 10 ml. in a volumetric flask, and the activity tested using 0·2 ml. The procedure was repeated using lower formaldehyde concentration.

(2) The rate of hydrolysis of BAEE in the absence of formaldehyde was followed over a 4 min. period. One ml. of 40% (w/v) formaldehyde at pH 7·6 was then added, the pH brought rapidly back to 7·6 and the rate measurements continued. This procedure was repeated using 2 ml. of 40% (w/v) formaldehyde.

(3) The rate of hydrolysis of 10 ml. of 0·02 M acetyl-L-phenylalanine ethyl ester (APEE) by 0·04 mg. of chymotrypsin was followed in the absence and presence of 2% (w/v) formaldehyde.

Effect of formaldehyde concentration on the observed amidase activity

The hydrolysis of 0·044 M-BAA was followed as described in the above method, except that 2·0 ml. of formaldehyde were used. 9·8 mg. of enzyme were used for each sample. The final concentration of BAA was adjusted to 0·044 M since K_m was, at this stage, undetermined.

RESULTS AND DISCUSSION

As can be seen from Fig. 1, the rate of hydrolysis of BAEE is unaffected by formaldehyde and the rate of observed BAA hydrolysis is insensitive to formaldehyde concentration. The rate of chymotrypsin-catalysed hydrolysis of acetyl-L-phenylalanine ethyl ester is materially reduced upon addition of formaldehyde. However, the residual activity appears to remain constant throughout the experiment; this probably indicates formation of a chemically altered but stable formol enzyme.

If S_0 is the original concentration of substrate at time t_0, S its concentration at time t, and E_0 the total concentration enzyme, the integrated form of the Michaelis equation is

$$(S_0-S)+K_m \ln S_0/S=k_3E_0(t_0-t). \qquad (1)$$

Examination of the curves in Fig. 2 indicates that the experimental plot of (S_0-S) against (t_0-t) deviates far more rapidly from linearity, especially at lower substrate concentrations, than is predicted by eqn. 1. This effect has been previously noted by Harmon & Niemann (1949), who ascribed it to a strong inhibition by product. Taking the concentra-

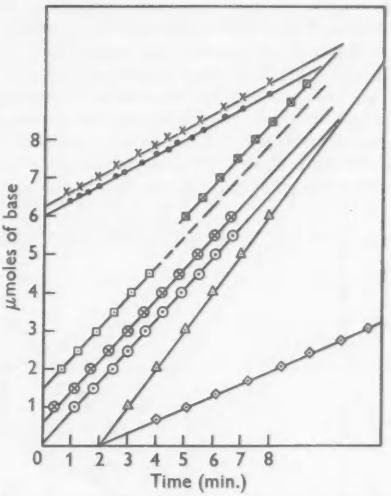

Fig. 1. Effect of formaldehyde on the rate of trypsin- (pH 7·60) and chymotrypsin- (pH 7·90) catalysed hydrolysis at 25°. 10 ml. of 3×10^{-3} M-BAEE in 0·01 M phosphate buffer and 0·2 M-NaCl solution containing: ⊙—⊙, no formaldehyde, 0·03 mg. of trypsin; ⊗—⊗, 0·03 mg. of trypsin previously treated with 20% (w/v) formaldehyde; ⊡—⊡, 0·03 mg. of trypsin and no formaldehyde for the first 4 min., then 4% (w/v) formaldehyde (⊠—⊠). 10 ml. of 0·044 M-BAA in 0·01 M phosphate buffer and 0·2 M-NaCl containing 3·3 mg. trypsin and the following concentrations of formaldehyde: ●—●, 2% (w/v); ×—×, 8% (w/v); 10 ml. of 0·02 M-APEE in 0·05 M ethylenediamine buffer and 0·2 M-NaCl containing 0·04 mg. chymotrypsin and: △—△, no formaldehyde; ◇—◇, 2% (w/v) formaldehyde.

tion of the product as $(S_0 - S)$ and K_I as the enzyme–product dissociation constant gives

$$(1 - K_m/K_I)(S_0 - S) + K_m(1 + S_0/K_I) \ln S_0/S = k_3 E_0(t_0 - t). \quad (2)$$

Eqn. 2 shows that the rate of hydrolysis would decrease with time more rapidly the smaller is K_m/K_I. During the very early part of the reaction, as followed in these experiments, $\ln S_0/S$ differs negligibly from $(S_0 - S)/S_0$ so that eqn. 2 becomes:

$$\{(1 - K_m/K_I) + K_m(1 + S_0/K_I)/S_0\}(S_0 - S) = k_3 E_0(t_0 - t). \quad (3)$$

Eqn. 3 shows that, on the hypothesis of inhibition by the product, $(S_0 - S)$ should be linear against t, irrespective of the value of K_I, during the earliest part of the reaction. This was found, in fact, not to be the case. A smooth curve could be fitted to the product/time points and from this curve the rate was interpolated at mean times. A plot of rate versus time was constructed from the curve as shown in Fig. 3. It can be seen that the rate decreases linearly with time, the deceleration being a function of the initial substrate concentration.

The linear deceleration of the hydrolysis can be attributed to the self-digestion of the enzyme at the pH optimum. It was for this reason that the rate could not be readily followed by any method involving the removal of samples over periods longer than 10 min. This self-digestion appears to be inhibited by substrate. When benzoyl-L-arginine ethyl ester was used as substrate in similar concentrations (0·01–0·003 M), the rate was found to be constant over longer intervals (up to 30 min.). However, when this substrate concentration was of the order of 3×10^{-5} M deviations from linearity became marked. This suggested the possibility that the self-digestion process was a function of the fraction of sites saturated with substrate. This is borne out by the upper plot shown in Fig. 3 where the deceleration/(rate at zero time) is the same for $9{\cdot}1 \times 10^{-3}$ M-BAA and $3{\cdot}1 \times 10^{-5}$ M-BAEE. $K_m/[S_0]$ is equal to 0·34 in both cases. K_m of trypsin for BAEE ($0{\cdot}96 \times 10^{-5}$ M) had been previously determined in this laboratory (Bernhard & Gutfreund, to be published).

K_m for the amide was determined by the usual method of Lineweaver & Burk (1934) at substrate concentrations between 10^{-2} and 10^{-3} M as illustrated in Fig. 4, and was found to be $3{\cdot}1 \times 10^{-3}$ M.

By extrapolation of the plot (Fig. 4) to infinite substrate concentration the maximum velocity was determined. $k_3[E_0] = 0{\cdot}94 \times 10^{-6}$ moles/mg. enzyme nitrogen min. Assuming a molecular weight of 17 000 for trypsin (Gutfreund, 1954), the first-order specific rate was calculated. $k_3 = 0{\cdot}043$ sec.$^{-1}$.

The fact that trypsin activity is unaffected by formaldehyde is partially explicable in terms of its specificity. There is evidence that a positive charge on the substrate contributes to the specificity

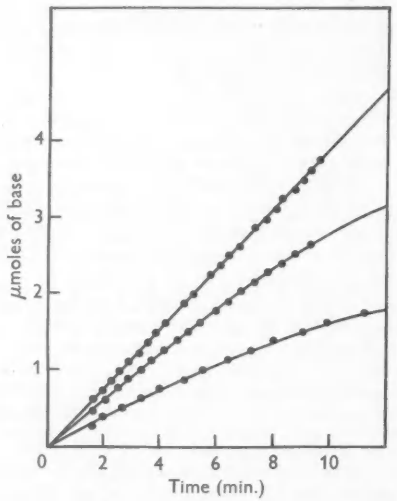

Fig. 2. Hydrolysis of benzoyl-L-arginineamide at pH 7·60 and 25° (in solutions in 0·01 M in phosphate buffer, 0·2 M in NaCl and containing 3·3 mg. of trypsin) at concentrations of $2{\cdot}96 \times 10^{-3}$ M (lower curve), $9{\cdot}1 \times 10^{-3}$ M (middle curve) and $4{\cdot}4 \times 10^{-2}$ M (upper curve).

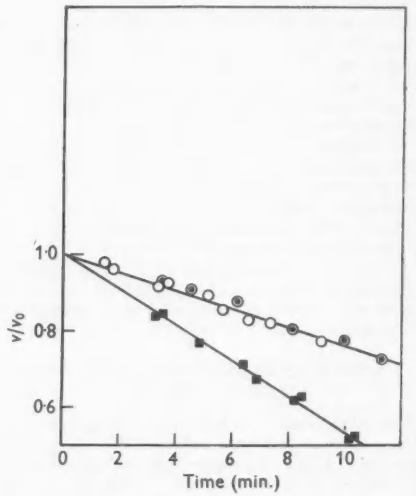

Fig. 3. Deceleration of the trypsin-catalysed hydrolysis in 0·2 M-NaCl at pH 7·60 and 25°. ■—■, $2{\cdot}96 \times 10^{-3}$ M-BAA, 0·01 M phosphate, 3·3 mg. trypsin; ⊙—⊙, $9{\cdot}1 \times 10^{-3}$ M-BAA, 0·01 M phosphate, 3·3 mg. trypsin; ○—○, $3{\cdot}1 \times 10^{-5}$ M-BAEE, 0·01 M phosphate, 0·016 mg. trypsin; v is the velocity at mean times obtained by point-to-point interpolation of product/time plots.

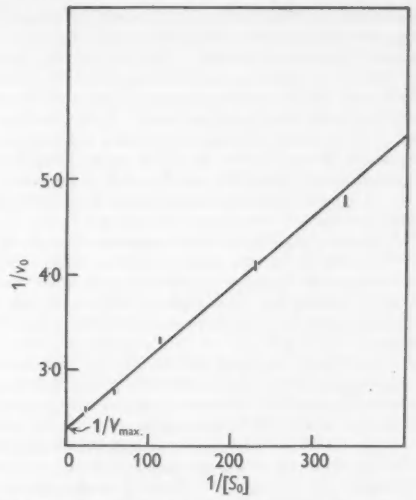

Fig. 4. Determination of K_m of benzoyl-L-arginineamide at pH 7·60 in 0·01 M phosphate buffer, 0·2 M-NaCl and 2% (w/v) formaldehyde. Each reaction mixture contained 3·3 mg. of enzyme. $[S_0]$ and v_0 are the respective initial substrate concentrations and rates. K_m was calculated from the slope by means of the equation

$$\frac{(V_{max.})_{[S]\to\infty}}{v_0} = \frac{K_m}{[S_0]} + 1.$$

(Neurath & Schwert, 1950). It would be unlikely, therefore, that a free amine group (ammonium ion at pH 7·6) would be found in the region of the active site. It is of interest to note that acetylation of trypsin results in no loss of esterase activity (Jansen, Nutting, Jang & Balls, 1949).

SUMMARY

1. Although formaldehyde alters the chemical structure of trypsin it has no effect on the activity of the enzyme at the pH optimum.

2. A method for following the trypsin-catalysed hydrolysis of amides based on the above observation has been described.

3. The enzyme–substrate dissociation constant for benzoyl-L-arginineamide has been determined ($K_m = 3·1 \times 10^{-3}$ M) as well as the first-order specific rate of decomposition of the enzyme–substrate complex ($k_3 = 0·043$ sec.$^{-1}$).

4. The pronounced deceleration of trypsin-catalysed hydrolysis with time has been explained in terms of the self-digestion of the enzyme (a process which is inhibited by the substrate).

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The Amide Groups of Insulin

BY F. SANGER,* E. O. P. THOMPSON† AND RUTH KITAI
Department of Biochemistry, University of Cambridge

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Previous work has shown that insulin consists of two types of polypeptide chains the amino acid sequences of which have been determined by partial hydrolysis experiments (Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*). For an assumed molecular weight of approximately 6000, which is the simplest chemical unit consisting of one phenylalanyl and one glycyl chain, there are six amide groups present (Rees, 1946). From a quantitative study of the products of reduction of insulin ester with lithium borohydride Chibnall & Rees (1952) showed that the three aspartic acids, one of which is

* Member of the scientific staff of the Medical Research Council.

† Present address: Wool Textile Research Laboratories, 343 Royal Parade Parkville, Victoria, Australia.

the *C*-terminal residue of the glycyl chain, are all present as amides, whereas three glutamic acid residues are amidized and the other four have free carboxyl groups. The following paper describes experiments to locate these amide groups on the individual dicarboxylic acid residues. To achieve this, use has been made of the fact that amide groups are split rapidly by the action of acid, but are not in general attacked by proteolytic enzymes. Thus peptides obtained from enzymic digests contain the original amide groups intact, whereas those obtained from acid hydrolysates do not. Two methods are thus available for identifying amide groups. In the first, the ionophoretic rates of the peptides from enzymic digests are compared with the rates of other similar peptides obtained from acid and enzymic hydrolysates. From these results it is possible to calculate the number of charged groups on the peptides and hence the number of carboxyl groups that are masked as amides. In the second method the number of amide groups present in the peptides from enzymic digests is determined directly by an estimation of the ammonia produced by hydrolysis with acid.

A number of peptides were identified from peptic and chymotryptic hydrolysates of fraction *A* (Sanger & Thompson, 1953*b*) and these were used to locate most of the amide groups. However, other peptides were required, especially those containing only one of the two glutamic acid residues in positions 4 and 5 (see Table 7), and so hydrolysates obtained by the action of two other enzymes (papain and mould protease) were investigated and the results are reported. The mould protease used was first isolated in a crystalline form by Crewther & Lennox (1950), but was not considered to be a pure substance. It is extremely active though rather unspecific in its attack.

The abbreviations and reference numbers to the peptides are those reported in previous papers (Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*). Pyr is used to denote a residue of pyrrolidone carboxylic acid (5-oxopyrrolidine-2-carboxylic acid).

EXPERIMENTAL

Action of mould protease on fraction A (*Expt. A*m). The mould protease used was a crystalline sample obtained through Dr R. R. Porter of the National Institute for Medical Research, Mill Hill, London.

A solution of fraction *A* (30 mg.) in 5 ml. water was brought to pH 8 and 1·5 mg. mould protease were added. After incubating at 37° for 24 hr. the mixture was boiled to inactivate the enzyme and taken to dryness *in vacuo*. Samples (5 or 10 mg.), after centrifuging to remove insoluble matter, were fractionated on Whatman no. 4 or no. 3 paper chromatograms, using phenol–0·3% NH_3 followed by butanol–acetic acid as solvents and the peptide spots eluted and subjected to hydrolysis and end-group determination as described in previous papers (Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*).

Action of papain on fraction A (*Expt. A*pa). The papain used was a commercial preparation supplied by British Drug Houses Ltd. A sample (16 mg.) was activated before use by incubation for 1 hr. with 1·6 ml. 1% NaCN solution at 37°. The activated enzyme (0·5 ml.) was then added to a solution of 50 mg. fraction *A* in 2 ml. water. The pH was adjusted to 7 with dilute NH_3 and the mixture incubated at 37° for 24 hr. Subsequent working up was as described for mould protease.

Preparation of peptide Ap5 by ionophoresis. Peptide *A*p5, which consists of the first thirteen residues of the glycyl chain (Sanger & Thompson, 1953*b*) was prepared from insulin by making use of its high mobility as an acid in 0·2N acetic acid, owing to its content of three cysteic acid residues.

Insulin (50 mg.) in 10 ml. 0·01N-HCl was treated with 1·0 mg. pepsin and incubated at 37° for 24 hr. After being taken to dryness, the residue was oxidized with performic acid (Sanger, 1949*a*). The formic acid was removed *in vacuo*, the residue dissolved in 1·5 ml. water and applied along the centre line of a sheet of Whatman no. 3 filter paper. It was then subjected to ionophoresis in an apparatus similar to that of Durrum (1950). A potential of 220v was applied for 21 hr. A strip was cut from the paper and tested with ninhydrin. The *A*p5 was present in a band which stained rather weakly with ninhydrin and had moved 12·5 cm. towards the anode. From a ninhydrin determination on a hydrolysate of the eluted material it appeared that the yield of *A*p5 was about 30% of the theoretical yield from insulin. In several experiments a faint slower moving band could be distinguished, which appears to be peptide *A*p9 (the first ten residues of the glycyl chain). For further purification the *A*p5 was subjected to ionophoresis at pH 3·7 in the apparatus of Michl (1951). The material obtained from 50 mg. insulin was put on a 18 cm.-wide strip of no. 3 filter paper; the ionophoresis was continued for 3·5 hr. at 1500v. The main *A*p5 band had moved 19 cm. towards the anode. Several fainter slow moving bands were also present.

Ionophoresis of peptides. The ionophoretic rates of peptides have been compared using the apparatus of Durrum (1950). The electrolyte used was 0·05M ammonium acetate (pH 6·8). The peptides obtained from paper chromatograms were applied to a sheet of Whatman no. 1 filter paper either directly by the 'strip transfer' method, or after elution from the paper and drying in a desiccator. The potential used was 220v; since the rates were not reproducible from experiment to experiment, only those done on the same paper were compared. If the original peptide spot had been treated with ninhydrin, the purple colouring matter usually migrated slightly towards the anode and was distinguished from the colour given by neutral spots on subsequent spraying, since they moved towards the cathode with the electroendosmotic flow.

Micro amide determinations on peptides. Estimations of amide N on the peptides eluted from paper chromatograms were carried out by the standard procedure (see Bailey, 1937), the ammonia being estimated by the micro-distillation method of Conway (1950). In order to determine the amount of peptide present in the samples, portions were subjected to hydrolysis, and the total amino acids estimated by the ninhydrin method of Moore & Stein (1948).

Samples of the peptides required for analysis were collected from several chromatograms on Whatman no. 3 filter paper, or from ionophoresis experiments. If the spots had been located with dilute ninhydrin, the 'cuts' were thoroughly washed in acetone before elution. The peptide sample was dissolved in a small amount of water and divided into portions which were transferred to small flat glass dishes or Polythene strips. In general, one-sixth was taken for estimation of the amount of peptide present and two one-third samples for amide estimation.

The smaller portion for estimation of total peptide was taken to dryness in a desiccator, dissolved in 5·7N-HCl, transferred to a capillary tube, and incubated at 105° for 24 hr. The hydrolysate was then transferred to Polythene and the excess HCl removed by repeated evaporation in a desiccator. In order to remove any NH_3, which would react with the ninhydrin reagent, 0·1 ml. 0·1M-K_2CO_3 was added to the residue on the Polythene strip, which was placed *in vacuo* for 2 hr. The NH_3-free hydrolysate was then dissolved in 5 ml. water and portions taken for estimation by the ninhydrin method using 1 ml. of ninhydrin reagent and a 30 min. reaction period (Moore & Stein, 1951).

The larger samples of the eluted peptides for amide estimations were taken to dryness, 0·1 ml. 0·1M-K_2CO_3 added and free NH_3 was removed by evaporation in a desiccator for 2 hr. The dry samples were then dissolved in 0·2 ml. 2N-HCl, transferred to capillary tubes and incubated at 105° for 3 hr. Reagent blanks were carried out simultaneously. The hydrolysed samples were divided into portions, each containing *ca.* 5 μg. NH_3-N, which was determined by the standard Conway method. 0·0005N-HCl was used in the centre compartment and back titration was carried out with 0·0025N-$Ba(OH)_2$.

RESULTS

Ionophoresis of peptic hydrolysate of fraction B (*B*p). In order to obtain samples of peptides from fraction *B* for amide estimations, 50 mg. of a peptic hydrolysate were fractionated by ionophoresis in 0·05M ammonium acetate using a 25 cm.-wide sheet of Whatman no. 3 paper. A number of clear bands were obtained and were cut out as shown in Fig. 1. Paper chromatography of hydrolysates of samples of the fractions indicated the composition shown and with the exception of band γ seemed to be reasonably pure.

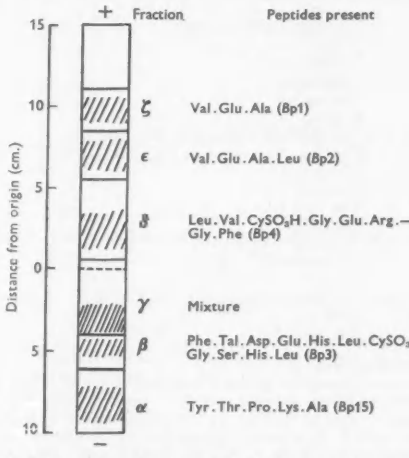


Fig. 1. Ionophoresis of peptic hydrolysate of fraction *B* in 0·05M ammonium acetate. 220v, 20 hr.

Action of mould protease on fraction A. The results obtained with the mould protease hydrolysates of fraction *A* are summarized in Fig. 2 and Table 1. All peptides were hydrolysed to identify the amino acids. In Tables 1–4 the approximate relative amount (strength) of the peptide spots is indicated in the second columns. This was determined by the strength of the ninhydrin colour reaction of the amino acids obtained on hydrolysis of the peptide. The ×'s have the same significance as in previous papers (Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*). The *N*-terminal residues of some of the peptides were determined by hydrolysis of their DNP-derivatives. The experimental results are summarized in column 3 (composition) and in column 4 are shown the most probable structures. These are deduced from a consideration of the data in column 3, the structure of fraction *A* and the known points of splitting by the enzyme, as determined from the structure of the other peptides present. Also the structures of some of the peptides have been inferred by comparison of their R_F values and ionophoretic rates with those of peptides of known structure.

Action of papain on fraction A (*A*pa). The results obtained with the papain hydrolysate are summarized in Fig. 3 and Table 2. Better resolution of the slower-moving peptides (spot nos. 1–9) was obtained by running both solvents two lengths of the paper. The results in Table 2 have the same significance as those in Table 1 and the probable structures were deduced in the same manner.

Peptide *A*pa1, which contained no free α-amino group, was detected by examination of the chromatogram in ultraviolet light. It was subjected to partial hydrolysis with 12N-HCl at 37° for 3 days and the peptides listed in Table 3 were identified. There was also present a spot (R_F in phenol, 0·7; R_F in butanol–acetic acid, 0·68) which gave no colour with ninhydrin, but gave glutamic acid on hydrolysis. This was most probably pyrrolidone carboxylic acid, which was shown to have these R_F values. This and the fact that peptide *A*pa1 contains no free amino group indicate that it is a pyrrolidone carboxylic acid derivative, and the partial hydrolysis experiments show that its structure is that given in Table 2.

No satisfactory explanation can be offered for the presence of spot *A*pa9, which does not fit into the structure of fraction *A*. On ionophoresis it moved

toward the anode, suggesting the glutamic acid residue is not present as an amide. The yellow colour with ninhydrin suggests that serine is *N*-terminal. It is tentatively suggested that synthesis may have occurred under the influence of the papain, but it is hoped to investigate this effect further.

Action of papain on Ap5. Peptide *Ap5* purified by a single ionophoresis, was hydrolysed with cyanide-activated papain as described above and the hydrolysate subjected to ionophoresis in 0·2M acetic acid overnight at 220v. Test strips were coloured with ninhydrin, and by the chlorination procedure of Rydon & Smith (1952). The distribution of the bands is shown in Fig. 4, and their composition in Table 4. Since band 5 gave no colour with ninhydrin but a strong colour by the chlorination method and contained glutamic acid, it is presumed to be a pyrrolidonoyl peptide. The presence of free tyrosine (band 2) is probably due to some impurity in the *Ap5*.

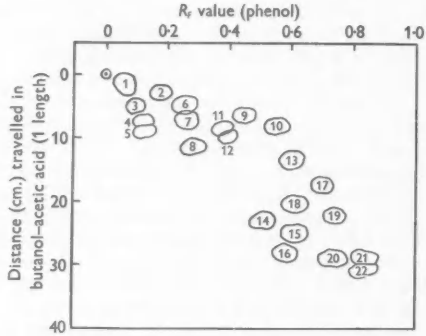


Fig. 2. Chromatogram of mould protease hydrolysate of fraction *A* (Expt. *Am*) (see Table 1).

Total amides of fraction A. Sanger (1949*a*) obtained a value of 15·15% for the amide-N of fraction *A* as percentage of the total N. This approximates to four residues per chain (theoretical 16·0%). Since it was essential to be certain of this value, it has been confirmed in the present work.

Bound NH_3 was removed before amide estimation by dissolving the fraction *A* (15–20 mg.) in 1 ml. 0·1M-K_2CO_3 and leaving in a vacuum desiccator over H_2SO_4 for 4 hr. The value obtained was 16·7% amide-N as percentage of total N. The theoretical value for five residues would be 19·2%.

Further evidence that there are four amide groups in fraction *A* was obtained from plotting a titration curve. Since fraction *A* contains no histidine, the only groups titrating in the range pH 2·5–7 are the

Table 1. *Peptides from mould protease hydrolysates of fraction A* (*Am*)

Spot no. (Fig. 2)	Approx. strength of peptide	Composition*	Probable structure
1	× × ×	Glu.[$CySO_3H$, Ser, Ala, Val]	Glu.$CySO_3H$.$CySO_3H$.Ala.Ser.Val.$CySO_3H$
2	× × ×	[$CySO_3H$, Ser, Ala, Val]	$CySO_3H$.Ala.Ser.Val.$CySO_3H$
3	×	[$CySO_3H$, Glu, Ser, Ala]	Mixture
4	× ×	[$CySO_3H$, Asp, Tyr]	Tyr.$CySO_3H$.Asp
5	×	[$CySO_3H$, Asp, Tyr]	—
6	× × × ×	[$CySO_3H$, Asp, Tyr]	Asp.Tyr.$CySO_3H$.Asp
7	×	[$CySO_3H$, Asp, Tyr]	—
8	×	—	Glutamic acid
9	× ×	[Asp]	Asparagine
10	× ×	[Glu]	Glutamine
11	× × ×	[Ser]	Serine
	×	[$CySO_3H$, Val]	Val.$CySO_3H$ and/or Ser.Val.$CySO_3H$
12	× ×	[$CySO_3H$, Glu, Gly, Val, Leu]	Gly.Ileu.Val.Glu.Glu.$CySO_3H$
13	×	—	Alanine
14	× × ×	Val.Glu	Val.Glu
15	× ×	Glu.[Glu, Leu]	Glu.Leu.Glu
16	× × ×	Leu.Glu	Leu.Glu
17	× ×	[Glu, Tyr]	Tyr.Glu
18	× ×	—	Tyrosine
19	×	[Glu, Val]	?
20	× ×	[Glu, Gly, Val, Leu]	Gly.Ileu.Val.Glu
21	× × ×	—	Leucine
22	× × ×	[Ser, Tyr, Leu]	Ser.Leu.Tyr

* Leucine and isoleucine were not distinguished. Both are recorded as Leu.

Spot 1. Faint colour with ninhydrin. *Spots* 1 *and* 2. May have an extra serine in the *C*-terminal position. *Spot* 3. Contained both glutamic acid and cysteic acid as *N*-terminal residues, so it was clearly a mixture. *Spot* 4. Grey colour with ninhydrin similar to tyrosine, suggesting tyrosine is *N*-terminal. *Spot* 6. Similar R_F value and colour with ninhydrin (yellow) to *Ap3* and *Apa3* (Asp.Tyr.$CySO_3H$.Asp). *Spots* 5 *and* 7. May be deamidated forms of *Am4* and *Am6* or one could be Asp.Tyr.$CySO_3H$. *Spot* 9. Yellow-brown with ninhydrin. *Spots* 12 *and* 22. Yellow with ninhydrin. *Spot* 20. On the chromatogram was a mixture with a tyrosine-containing peptide. It was purified by ionophoresis, yellow colour with ninhydrin. Same R_F value as peptide *A*1γ3 (Gly.Ileu.Val.Glu).

Table 2. *Peptides from papain hydrolysate of fraction A* (*A*pa)

Spot no. (Fig. 3)	Approx. strength of peptide	Composition*	Probable structure
1	× × ×	[$CySO_3H$, Glu, Ser, Ala, Val]	Pyr.$CySO_3H$.$CySO_3H$.Ala.Ser.Val.$CySO_3H$
2	× ×	[$CySO_3H$, Asp, Glu, Tyr]	Glu.Asp.Tyr.$CySO_3H$.Asp
3	× ×	[$CySO_3H$, Asp, Tyr]	Asp.Tyr.$CySO_3H$.Asp
4	×	[$CySO_3H$, Asp, Tyr]	—
5	× × ×	[$CySO_3H$, Asp, Glu, Tyr, Leu]	Leu.Glu.Asp.Tyr.$CySO_3H$.Asp
6	×	[$CySO_3H$, Asp, Glu, Tyr, Leu]	—
7	×	—	Glutamic acid
8	× ×	—	Glycine
9	×	[Glu, Ser, Tyr]	?
10	× ×	—	Tyrosine
11	× × ×	[Glu, Tyr]	Tyr.Glu
12	×	[Glu, Tyr, Leu]	Tyr.Glu.Leu.Glu
13	×	[Glu, Ser, Tyr, Leu]	Ser.Leu.Tyr.Glu
14	×	[Glu, Tyr, Leu]	Tyr.Glu.Leu
15	× × × ×	[Glu, Gly, Val, Leu]	Gly.Ileu.Val.Glu
16	× × ×	Leu, Glu	Leu.Glu
17	× ×	[Glu, Val, Leu]	Ileu.Val.Glu
18	× ×	[Ser, Leu]	Ser.Leu
19	× ×	[Glu, Leu]	Pyr.Leu

* Leucine and isoleucine were not distinguished. Both are recorded as Leu.

Spot 1. No colour with ninhydrin. Located by slight fluorescence in ultraviolet light. It gave no DNP derivative, indicating no free —NH_2 group. *Spot* 2. R_F values and ionophoresis rates identical with those of peptide *A*p1. *Spot* 3. Yellow with ninhydrin. R_F values and ionophoretic rates identical with those of peptide *A*p3. *Spots* 4 *and* 6. Only detected when solvents run 2 lengths. May be deamidated forms of 3 and 5 or may have other structures. *Spot* 5. R_F values and ionophoretic rates identical with those of peptide *A*p6. *Spot* 8. Gives traces of serine, valine and cysteic acid after hydrolysis. *Spots* 9, 13 *and* 18. Yellow with ninhydrin. *Spot* 10. Corresponds in composition to spots *A*p12 and *A*m18 while spot 11 corresponds to spots *A*p11 and *A*m17, but the rates of movement in phenol and butanol–acetic acid were reversed. *Spot* 12. Obtained from the chromatogram was contaminated with spot 15, from which it could be separated by ionophoresis. Is probably identical with *A*p14. *Spot* 14. Probably identical with *A*p15. *Spot* 15. Strong grey colour with ninhydrin. *Spot* 19. No visible colour with ninhydrin. Located by fluorescence. Its R_F value in phenol is greater than the known peptides of leucine and glutamic acid. It is probably a pyrrolidone carboxylic acid derivative formed from an *N*-terminal glutamine residue.

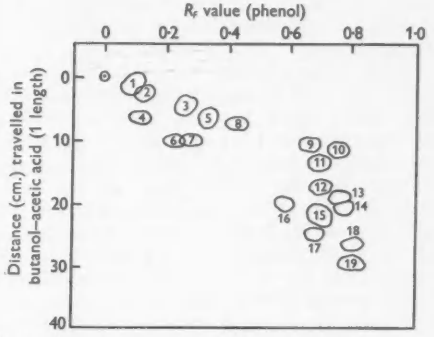


Fig. 3. Chromatogram of papain hydrolysate of fraction *A* (Expt. *A*pa) (see Table 2).

Table 3. *Partial hydrolysis products of peptide A*pa1

Peptide	Strength
$CySO_3H$.$CySO_3H$.Ala (*A*2δ1)	× ×
Cysteic acid	× ×
Glu.$CySO_3H$ (*A*1α1)	×
$CySO_3H$.Ala (*A*1γ3)	× ×
Glutamic acid	× ×
Ser.Val.$CySO_3H$ (*A*1ζ2)	× × × ×
Serine	×
Alanine	× × ×

carboxyl groups. The —SO_3H groups are considerably stronger and are probably completely ionized in this range. Fraction *A* (20·1 mg.) from which the bound acetate had been removed by washing well with 0·1 N-HCl in acetone, was titrated to pH 2·27 with 0·025 N-HCl. It was then titrated back to pH 7 with 0·025 N-NaOH, taking the usual precautions. The results (Fig. 5) are corrected for the activity of HCl in water. Although there was considerable scatter at the lower pH values, it is evident that there are three free —COOH groups on fraction *A*. One of these is the free α-COOH group, so that two of the six dicarboxylic amino acids have free ω-COOH groups and the other four are present as amides.

Ionophoretic rates of peptides. Table 5 shows the distance moved by the various peptides on ionophoresis in 0·05 M ammonium acetate. In this table the results listed in each column were obtained in the same experiment and so are directly comparable. Results from different experiments are not strictly comparable, although in general a peptide moving slightly towards the cathode is neutral and peptides moving towards the anode are acidic.

Table 6 shows the results of the amide determinations on the peptides produced by enzymic hydrolysis of fractions *A* and *B*.

Table 4. *Action of papain on peptide Ap5 (Ap5pa)*

Band no. (Fig. 4)	Strength	Composition	Probable structure
1	× × ×	[Glu, Gly, Val, Leu or Ileu]	Gly.Ileu.Val.Glu
2	×	[Tyr]	Free tyrosine
3	× × ×	[$CySO_3H$, Ser, Val, Leu or Ileu]	Val.$CySO_3H$.Ser.Leu
4	×	[$CySO_3H$, Glu, Ser, Gly, Ala, Val, Leu or Ileu]	Unchanged *Ap5*
5	× × × ×	[$CySO_3H$, Glu, Ser, Ala]	Pyr.$CySO_3H$.$CySO_3H$.Ala.Ser

Band 3. Also contained traces of glutamic acid.

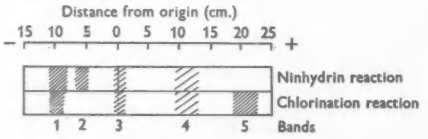


Fig. 4. Ionophoresis of papain hydrolysate of peptide *Ap5* in 0·2M acetic acid, 220v, 16 hr. (Expt. *Ap5pa*) (see Table 4).

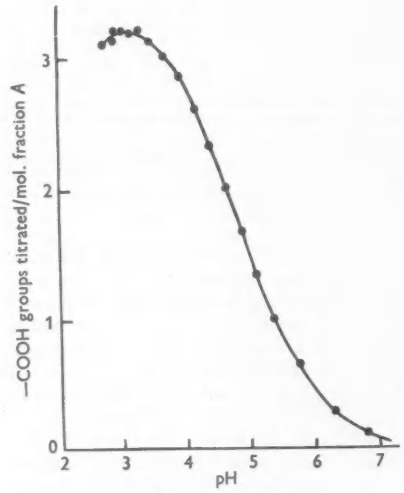


Fig. 5. Titration curve for fraction *A*.

DISCUSSION

Amide groups in the phenylalanyl chain

The amide contents of the peptides from fraction *B* (Table 6) were obtained using the fractions obtained by ionophoresis (Fig. 1), which may not have been completely pure. There may therefore be some error in the results but they are sufficiently accurate for the present purposes.

Sanger (1949*b*) showed that the aspartic and glutamic acid residues in positions 3 and 4 respectively were both in the form of amides, since several different *N*-terminal DNP-peptides containing the same amino acids could be obtained. This is supported by the amide content of peptide *Bp3*, which gives a value approximating to the expected two residues.

Sanger & Tuppy (1951*b*) concluded that the glutamic acid residue in position 13 (see Table 7) contained a free γ-COOH group, since peptides *Bp1* (Val.Glu.Ala+Val.Glu) and *Bp5* (Leu.Val.Glu) from the peptic hydrolysates had the same R_F values as the corresponding peptides from the acid hydrolysates. This is confirmed by the fact that the peptides *Bp1* and *Bp2* (Val.Glu.Ala.Leu) are acidic (Fig. 1) and contain no amide groups (Table 6).

The remaining glutamic acid residue (position 21) is present in peptide *Bp4* (Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly.Phe). This is slightly acidic (Fig. 1) and contains no amide groups, so that the glutamic acid residue must have a free γ-COOH group.

Amide groups in the glycyl chains

It has already been shown that the *C*-terminal residue is asparagine (Harris, 1952; Sanger & Thompson, 1953*b*) and this is confirmed by the presence of one amide group in peptide *Ac1* ($CySO_3H$.Asp).

Several of the peptides obtained from the peptic hydrolysate have duplicate spots which contain the same amino acids, but are more acidic. Thus peptides *Ap10* (Tyr.Glu), *Ap4* (Asp.Tyr.$CySO_3H$.Asp), *Ap2* (Glu.Asp.Tyr.$CySO_3H$.Asp) and *Ap7* (Leu.Glu.Asp.Tyr.$CySO_3H$.Asp) are the corresponding forms of peptides *Ap11*, *Ap3*, *Ap1* and *Ap6* respectively. It was at first thought that this was due to deamidation occurring under the action of pepsin, but no loss of amide-N could be detected from fraction *A* when it was incubated with pepsin. More probably the insulin used was already partially deamidated, since Harfenist (1953) has shown that certain preparations contain a component having one less amide group than the normal insulin.

Peptides *Ap11* and *Am17* both contain the sequence Tyr.Glu and are neutral. They are therefore Tyr.GluNH_2 and the glutamic acid residue in position 15 is present as an amide. There is considerable confirmation for this. Peptides *Ap15*, *Apa14* (Tyr.Glu.Leu) are also neutral and *Ap15* contains one amide group. Peptide *Am15* (Glu.Leu.Glu) moves more slowly on ionophoresis than Leu.Glu (*Am16*) and must therefore be GluNH_2.Leu.Glu.

Table 5. *Ionophoretic rates of peptides in* 0·05 M *ammonium acetate*

The figures listed in each column represent the results obtained in a single experiment.

Peptide	Spot no.	Distance moved towards anode (cm.) (15)*	(10)*	(15)*	(15)*	(14)*	(8·5)*	(15)*	(18)*	(18)*
Tyr.Glu	*Ap*11	—	−1	−2	—	—	—	—	—	—
	*Ap*10	—	—	5·5	—	—	—	—	—	—
	*Am*17	—	—	—	—	—	−1	—	—	—
Tyr.Glu.Leu	*Ap*15	—	−1	−2	—	—	—	—	—	—
	*Apa*14	—	—	—	—	—	—	−1	—	—
Tyr.Glu.Leu.Glu	*Ap*14	—	4·4	—	—	—	—	—	—	—
	*Apa*12	—	—	—	—	—	—	3·6	—	8
Leu.Glu	*Apa*16	—	—	—	—	—	—	6	—	—
	*Am*16	—	—	—	—	8·5	—	—	—	—
Glu.Leu.Glu	*Am*15	—	—	—	—	7	—	—	—	—
Asp.Tyr.$CySO_3H$.Asp	*Ap*3	8·7	7	—	8·2	—	—	—	—	—
	*Ap*4	15	—	14	—	—	—	—	—	—
	*Apa*3	—	—	—	8	—	—	—	—	—
Glu.Asp.Tyr.$CySO_3H$.Asp	*Ap*1	12·3	9	—	11·6	—	—	—	—	—
	*Ap*2	18·7	—	—	—	—	—	—	—	—
	*Apa*2	—	—	—	11·7	—	—	—	—	—
Leu.Glu.Asp.Tyr.-$CySO_3H$.Asp	*Ap*6	—	—	9	9·5	—	—	—	—	—
	*Ap*7	15	—	—	—	—	—	—	—	—
	*Apa*5	—	—	—	9·7	—	—	—	8	—
Gly.Ileu.Val.Glu	*Ap*16	—	—	4·3	—	—	—	—	—	—
	*Apa*15	—	—	—	—	—	—	4·3	—	—
	*Am*20	—	—	—	—	—	5	—	—	—
Ileu.Val.Glu	*Apa*17	—	—	—	6	—	—	—	—	—
Val.Glu	*Am*14	—	—	—	9	9·5	—	—	—	—
Ser.Leu.Tyr.Glu	*Apa*13	—	—	—	—	—	—	−1	—	—
Ser.Leu.Tyr	*Am*22	—	—	—	—	—	−1	—	—	—
Glu.Leu.Glu.Asp.Tyr.-$CySO_3H$.Asp	*Ac*3	—	—	—	—	—	—	—	6·7	—
Glu.Leu.Glu.Asp.Tyr	*Ac*5	—	—	—	—	—	—	—	—	6·7
Glutamine	*Am*10	—	—	—	—	−1	—	—	—	—
Asparagine	*Am*9	—	—	—	—	−1	—	—	—	—
Glutamic acid	—	14·5	—	16	—	—	—	—	—	—

* Time of ionophoresis (hr.).

Table 6. *Amide determinations of peptides*

Peptide	Sequence	No. of estimations averaged	Amide groups (residues/mol.)
*Bp*1	Val.Glu.Ala	2	0·15
*Bp*2	Val.Glu.Ala.Leu	2	0·15
*Bp*4	Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly.Phe	3	0·13
*Bp*3	Phe.Val.Asp.Glu.His.Leu.$CySO_3H$.Gly.Ser.His.Leu	2	1·7
*Ac*1	$CySO_3H$.Asp	1	0·7
*Ap*15	Tyr.Glu.Leu	2	0·98
*Ap*14	Tyr.Glu.Leu.Glu	2	1·03
*Ap*3	Asp.Tyr.$CySO_3H$.Asp	4	2·10
*Ap*1	Glu.Asp.Tyr.$CySO_3H$.Asp	7	1·94
*Ap*5*pa*1	Gly.Ileu.Val.Glu	2	0·15
*Ap*5	Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.Ala.Ser.Val.-$CySO_3H$.Ser.Leu	17	1·16

Similarly, Glu.Leu.Glu.Asp.Tyr.$CySO_3H$.Asp (*Ac*3) moves slower than Leu.Glu.Asp.Tyr.-$CySO_3H$.Asp (*Apa*5).

In the chymotryptic digests two peptides (*Ac*5, *Ac*7) were isolated having the same amino acid composition but one (*Ac*7) gave no colour with ninhydrin. It was pointed out that this was presumably due to the lability of an *N*-terminal glutaminyl residue which tends to cyclize to a pyrrolidone carboxylic acid residue. Thus *Ac*5 is probably $GluNH_2$.Leu.Glu.Asp.Tyr and *Ac*7 Pyr.Leu.Glu.Asp.Tyr. Also Pyr.Leu (*Apa*19) was found in the papain hydrolysate.

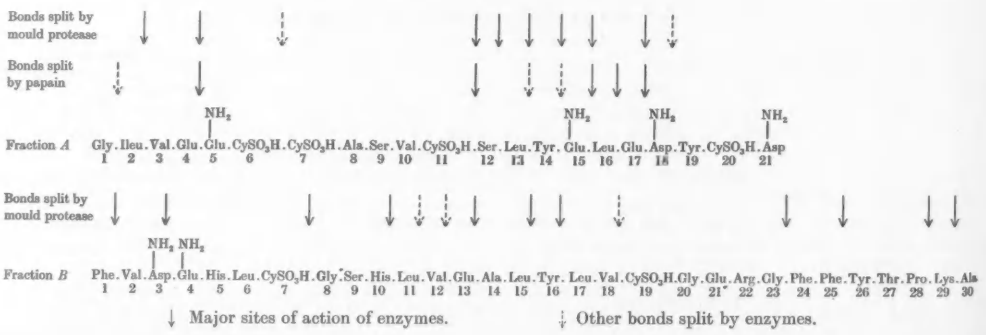
Whereas peptide *Ap*15 (Tyr.$GluNH_2$.Leu) was found to be neutral, peptide *Ap*14 (Tyr.Glu.Leu.-Glu) is acidic, which suggests the second glutamic acid residue has a free carboxyl group. This is supported by the fact that Leu.Glu (*Apa*16, *Am*16) and Glu.Leu.Glu (*Am*15) are acidic. The glutamic acid residue in position 17 therefore contains a free γ-COOH group. Amide determination also showed that peptide *Ap*14 (Tyr.$GluNH_2$.Leu.Glu) had only one amide group, as did *Ap*15 (Tyr.Glu-NH_2.Leu). Peptides *Ap*1 and *Apa*2 (Glu.Asp.Tyr.-$CySO_3H$.Asp) differ from peptides *Ap*3 and *Apa*3 (Asp.Tyr.$CySO_3H$.Asp) only by one glutamic acid residue but are more acidic on ionophoresis, which confirms that this glutamic acid residue (position 17) is not amidized. Similarly peptide *Ap*6 (Leu.Glu.-Asp.Tyr.$CySO_3H$.Asp) is less acidic than *Ap*1 and more acidic than *Ap*3.

Peptide *Ap*3 (Asp.Tyr.$CySO_3H$.Asp) contains two amide groups (Table 6). These can only be located on the aspartic acid residues so that both (positions 18 and 21) must be present as asparagine. Similarly *Ap*1 (Glu.Asp.Tyr.$CySO_3H$.Asp) contains two amide residues, and the glutamic acid residue has a free γ-COOH group. Further evidence for an amide in position 18 is that Glu.Leu.Glu.-Asp.Tyr (*Ac*5) moves slower on ionophoresis than Tyr.Glu.Leu.Glu (*Ap*14). These two peptides differ only by the presence of the aspartic acid residue in question, although they contain a different tyrosine residue.

Three enzymes have split the bond between the two glutamic acid residues in positions 4 and 5. Thus, papain gave a high yield of peptide *Apa*15 (Gly.Ileu.Val.Glu). On ionophoresis it moved towards the anode, showing that the glutamic acid was not present as an amide. Similarly, *Ap*16 and *Am*20, which have the same sequence as *Apa*15 are acidic and NH_3 determination showed there was no amide in *Ap*5*pa*1. Peptides *Apa*17 (Ileu.Val.Glu) and *Am*14 (Val.Glu) are also acidic. Peptide *Ap*5*pa*5 was identified as a pyrrolidonoyl peptide (Pyr.$CySO_3H$.$CySO_3H$.Ala.Ser) indicating that the residue in position 5 is glutamine.

From these results it was expected that peptide *Ap*5 (Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.-Ala.Ser.Val.$CySO_3H$.Ser.Leu) would contain one amide group (on position 5). However, innumerable determinations gave values which varied somewhat but were considerably closer to two than to one. Also a few determinations on peptides *Ap*7, *Ac*2 and *Ac*4 gave similar high results. The *Ap*5 was prepared either from chromatograms or more often by a single ionophoresis in 0·2 M acetic acid. Although the apparent absence of aspartic acid and tyrosine from hydrolysates suggested that the *Ap*5 was reasonably pure, it now seems that the high amide value was due to the presence of an amide-rich impurity, since when *Ap*5 was further purified by high-voltage ionophoresis at pH 3·7, values approximating to one were obtained. The figures averaged in Table 6 are all for such purified material. This impurity appears to travel slightly slower than *Ap*5 in the 0·2 M acetic acid ionophoresis since a cut taken behind the *Ap*5 band gave a higher amide value than the main band. It seems probable that it is largely unchanged fraction *A* and possibly

Table 7. *Amide distribution in insulin*


Bonds split by mould protease
Bonds split by papain
Fraction A
Gly.Ileu.Val.Glu.Glu.CySO3H.CySO3H.Ala.Ser.Val.CySO3H.Ser.Leu.Tyr.Glu.Leu.Glu.Asp.Tyr.CySO3H.Asp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
NH2
Bonds split by mould protease
Fraction B
Phe.Val.Asp.Glu.His.Leu.CySO3H.Gly.Ser.His.Leu.Val.Glu.Ala.Leu.Tyr.Leu.Val.CySO3H.Gly.Glu.Arg.Gly.Phe.Phe.Tyr.Thr.Pro.Lys.Ala
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
↓ Major sites of action of enzymes.
↓ Other bonds split by enzymes.

other similar large peptides. Fraction *A* has a comparatively high amide content and a small amount present in the *A*p5 would cause considerable error. In our experience it forms very long spread-out streaks both on ionophoresis and chromatography and, if present, would be expected to contaminate *A*p5 and other peptides (*A*p9, *A*c2, *A*c4) which move at similar rates.

On the basis of these combined results it is concluded that the distribution of amide groups in insulin is as shown in Table 7. This agrees well with the results of Chibnall & Rees (1952), who showed that all three aspartic acid and three of the glutamic acid residues are amidized.

The possibility of iso*glutamine residues*

Kandel, Kandel, Kovacs & Bruckner (1954) have recently suggested that insulin contains an *iso*-glutamine residue. This is based on the finding that when insulin is subjected to the Hofmann degradation and hydrolysed, a substance is produced which gives a 2:4-dinitrophenylhydrazone that moves at the same rate on a butanol–3 % NH_3 paper chromatogram as the dinitrophenylhydrazone of 4-oxobutyric acid. This product would be obtained if an *iso*glutaminyl residue were present in the protein. We have repeated the above experiment and have confirmed the presence of a spot that moves on a butanol–3 % NH_3 chromatogram at the same rate as the synthetic dinitrophenylhydrazone of 4-oxobutyric acid. There were, however, rather more spots on the chromatogram than the two recorded by Kandel *et al.* Samples of the dinitrophenylhydrazone spot were eluted from the paper and reinvestigated by chromatography on *tert.*-amyl alcohol–phthalate (pH 6) buffer (Blackburn & Lowther, 1951) and in the 'toluene' solvent of Biserte & Osteux (1951). With the former solvent two strong spots were produced which had moved 6 and 11 cm. down the paper respectively and a fainter spot that had moved 19 cm. A control spot of the synthetic hydrazone had moved 19 cm. Similar results were obtained with the 'toluene' solvent. It thus seems that the spot that was identified by Kandel *et al.* as the above hydrazone, was in fact a mixture of several substances, one of which may have been the hydrazone. It is present in only small quantities and would probably account for about 1–2 % of one residue in insulin (mol.wt. 5700). In view of these results and the known complexity of the reactions of hypochlorite with amino acids, peptides and proteins (see e.g. Goldschmidt & Strauss, 1930 and previous papers) it seems that there is no clear evidence for the existence of *iso*glutamine residues in insulin.

The fact that the residues in positions *A*5 and *A*15 are converted to a pyrrolidonoyl group when they are the *N*-terminal residues of a peptide (e.g. in peptides *A*pa1 and *A*c7, respectively) indicates that they are glutaminyl residues. An *iso*glutaminyl residue would not decompose to a pyrrolidonoyl peptide but would give the amide of pyrrolidone carboxylic acid. No such evidence exists for the residue in position *B*4, but a study of the Hofmann degradation of fractions *A* and *B* of oxidized insulin showed that the spot moving at the same rate as the dinitrophenylhydrazone of 4-oxobutyric acid was produced from both fractions.

Action of the proteolytic enzymes

The main sites at which splitting occurred under the action of papain and mould protease are indicated in Table 7. The sites of action of mould protease on fraction *B* are based on results of a single experiment and should not be regarded as conclusive and are not comprehensive. Both enzymes show a wide specificity which is somewhat similar to that of pepsin, though the relative intensities of splitting of the various bonds was markedly different for the three enzymes. Mould protease carried the digestion of fraction *A* further than the other enzymes. For structural studies in the future, this enzyme may prove useful, for example, in degrading longer chains obtained by the use of other enzymes.

It is doubtful if the preparations of the two proteinases represent pure enzymes. In the case of papain we have also studied the action of an activated sample of crystalline mercuripapain prepared by Kimmel & Smith (1954). The specificity appeared to be essentially the same as that found with the crude commercial sample.

It is interesting that papain breaks peptide *A*p5 in a different way from fraction *A*. In fraction *A* strong splitting occurs at the $CySO_3H$.Ser bond (position 11–12), whereas this bond was stable in *A*p5 and splitting occurred largely at the Ser.Val bond (position 9–10). This result could be explained if papain was inhibited by a free carboxyl group on the second residue from the bond. Other explanations are possible, but it does illustrate that the specificity of proteolytic enzymes is not conditioned solely by the nature of the residues involved in the bond in question.

SUMMARY

1. Fraction *A* of oxidized insulin was hydrolysed with mould protease and cyanide-activated papain and the resulting peptides studied. Both enzymes showed a rather wide specificity.

2. The positions of the amide groups of insulin were determined by estimating the relative ionophoretic mobilities and the amide contents of peptides from enzymic hydrolysates of fractions *A* and *B* of oxidized insulin. The results are summarized in Table 7.

We wish to thank Dr R. R. Porter for the mould protease and Dr Emil L. Smith for the crystalline mercuripapain.

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Metabolism of Polycyclic Compounds

8.* ACID-LABILE PRECURSORS OF NAPHTHALENE PRODUCED AS METABOLITES OF NAPHTHALENE

By E. BOYLAND and J. B. SOLOMON
Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, S.W. 3

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Baumann & Herter (1877) observed that crystals of naphthalene appeared in the distillate when the acidified urine of rabbits dosed with naphthalene was heated. Bourne & Young (1933) noticed that crystals of naphthalene separated from such acidified urine in the cold. They suggested that this might be due to the dehydration of 1:2-dihydro-2-naphthol, which is known to give naphthalene with cold mineral acid (Bamberger & Lodter, 1895), but dihydronaphthol does not appear to have been isolated from urines of animals dosed with naphthalene. Chang & Young (1943) and Young (1947) estimated that the acid-liberated naphthalene accounted for from 4 to 14 % of the dose fed to rats or rabbits. The liberation of anthracene on acidification of the urine of rats and rabbits dosed with anthracene was noticed by Boyland & Levi (1936). Chang & Young (1943) also found that small amounts of hydrocarbons were liberated on acidification of urines of rats dosed with phenanthrene and anthracene. Acid-labile precursors could not be detected in the urines of rats dosed with higher polycyclic hydrocarbons.

Studies on 1:2:3:4-tetrahydronaphthalene (tetralin) metabolism by Pohl & Rawicz (1919) and by Rockemann (1922) indicated that tetralin was converted into 1- or 2-tetralyl glucosiduronic acids by dogs and rabbits. When these tetralyl glucosiduronic acids were steam-distilled from acid solution, 1:2-dihydronaphthalene and naphthalene were produced. Pohl & Rawicz (1919) also dosed rabbits with 1:2-dihydronaphthalene and found that naphthalene was produced on acidification of the urine; they suggested that a 1:2-dihydronaphthyl glucosiduronic acid had been formed. Corner, Billett & Young (1954) have recently shown that 1:2-dihydronaphthalene-1:2-diol is excreted as a glucosiduronic acid derivative by rabbits dosed with naphthalene.

Evidence that naphthalene is metabolized to 1:2-dihydro-1-naphthyl glucosiduronic acids, which readily yield naphthalene in acid solution, is presented in this paper.

EXPERIMENTAL

Fifty rats (average body weight 250 g.) maintained on rat cake and water, were each injected intraperitoneally with naphthalene (100 mg.) dissolved in arachis oil (1 ml.) on

* No. 7 of this series: Boyland, E. & Wiltshire, G. M. (1953).

5 consecutive days. Four rabbits (body weight approximately 2 kg.) maintained on oats, rat cake, bread and water, were each dosed *per os* with naphthalene (1 g.) dissolved in arachis oil (10 ml.) on 4 consecutive days. The animals were housed in metabolism cages and the urine was collected during the period of dosing, and for 2 days after the last dose.

The melting points given in this paper are uncorrected.

Detection of naphthalene precursors

Samples of naphthalene urine were made alkaline and steam distilled for 5 min. to remove free naphthalene. The solutions were acidified (pH 2) and slowly steam distilled, when crystalline naphthalene separated in the distillate. The naphthalene precursor was also detected by paper chromatography. Solutions were applied to Whatman no. 1 (special chromatographic) paper which was developed downwards with the upper layer of a *n*-butanol–ethanol–water mixture (17:3:20) for 16 hr. This system separated the naphthalene precursor (approximate R_F 0·22), 1-naphthyl glucosiduronic acid (R_F 0·15) and 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid (R_F 0·08). The naphthalene precursor was seen on paper as a dark absorbent region under ultraviolet light (mainly 2536 Å) from a Hanovia chromatolite (Chance OX7 filter).

Isolation of naphthalene precursors

The 'naphthalene urine' was centrifuged to remove solid material and evaporated under reduced pressure to a brown gum which was extracted repeatedly with boiling ethanol until the extracts were colourless, and the residue became granular. The ethanolic extracts were cooled, filtered and evaporated under reduced pressure to a brown gum which was dissolved in a small volume of water. A mixture of equal parts by weight of charcoal (activated, British Drug Houses Ltd.) and kieselguhr was stirred with water to form a slurry, which was poured into a glass tube (diameter 3 cm.) with a sintered disk covered with sand (about 1 cm.) as a support for the adsorbent. The column (15 cm. long) was washed with water (1 l.). The aqueous solution of the gum (20 ml.), which gave a positive naphthoresorcinol reaction, was allowed to percolate down the column. The column was treated with distilled water (*ca.* 4 l.) using mild suction, until all the naphthalene precursor was eluted. This eluate (*A*) also contained urea, inorganic salts and a little 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid. When the charcoal column was subsequently eluted with 50% (v/v) aqueous acetone, a fraction (*B*) which contained mainly this glucosiduronic acid was obtained.

The aqueous eluate *A* was concentrated under reduced pressure and extracted with hot dioxan, which removed large quantities of urea and traces of 1:2-dihydronaphthalene-1:2-diol (1:2-diol), 1-naphthyl sulphuric acid and another unidentified metabolite of naphthalene. The residual gum was dissolved in water (2 ml.) and passed through a cellulose partition column prepared by carefully dry-packing (Hough, Jones & Wadman, 1949) Whatman cellulose powder into a glass tube (27 × 7 cm.) and equilibrating by washing with 1 l. of *iso*propanol–water (9:1). When the top of the column was dry, the solution containing the glucosiduronic acids was poured on, and allowed to soak in before development with *iso*propanol–water (9:1). The naphthalene precursor was present in the first three fractions (100 ml. each); continued development with this solvent did not elute 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid which was eluted with *iso*propanol–water (8:2). The fractions containing the naphthalene precursor were evaporated under reduced pressure to a clear yellow gum, which gave a strongly positive naphthoresorcinol test, effervesced in $NaHCO_3$ solution, produced naphthalene on acidification with mineral acid and did not couple with diazotized *p*-nitraniline. This gum still contained urea and other unidentified impurities and could not be crystallized from any of a number of solvent mixtures. As no water-insoluble salt of the glucosiduronic acid could be obtained, the clear gum was dissolved in hot methanol (20 ml.), cooled, filtered and treated with an excess of diazomethane. Removal of the solvent under reduced pressure afforded the methyl ester of the glucosiduronic acid as a brown gum, which was dissolved in hot methanol (10 ml.) and treated with a mixture (50 ml.) of equal volumes of pyridine and acetic anhydride. After 30 min. the mixture was poured into water and allowed to stand at 4° overnight, when a white powder separated, which was crystallized from warm aqueous ethanol as small needles which were dried *in vacuo* over P_2O_5 at 100°. Yields of this derivative from rat and rabbit urines were 100 mg. (0·4% dose) and 80 mg. (0·5% dose) respectively. This appeared to be *methyl* (1:2-*dihydro*-1-*naphthyl tri*-O-*acetylglucosid*)-*uronate*. The derivative isolated from rat urine had m.p. 203°; $[\alpha]_D^{22} - 84 \pm 3°$ in $CHCl_3$ (*c*, 1). (Found: C, 59·9; H, 6·1. $C_{32}H_{36}O_{16}$ requires C, 59·7; H, 5·7%.) And that from rabbit urine had m.p. 203°, unchanged by admixture with the rat derivative, and $[\alpha]_D^{22} + 23 \pm 1°$ in $CHCl_3$ (*c*, 1). (Found, C, 59·8; H, 5·9%.) Both derivatives were soluble in $CHCl_3$, acetone, ethyl acetate and warm ethanol but were insoluble in water. Comparative data for triacetyl methyl esters of the glucosiduronic acids of the 1:2-diol and 1- and 2-naphthol (Corner *et al.* 1954) are given in Table 1.

The rat and rabbit triacetyl methyl esters (5 mg. each) were separately shaken with water at pH 2 for 16 hr. Each solution was then made alkaline and steam distilled, when naphthalene collected in the condenser. The naphthalene was washed into the steam distillate with ethanol until the distillate contained 60% ethanol (100 ml.) and estimated spectrophotometrically at 275 mμ. with a Unicam spectrophotometer. The derivative obtained from rat urine yielded 1·0 mg. naphthalene and that from the rabbit urine 1·1 mg. naphthalene (theory 1·38 mg.). The residual solution after steam distillation from acid gave a strongly positive

Table 1. *Properties of certain glucosiduronic acid derivatives*

Derivative	Animal	$[\alpha]_D^{22}$ (°)	M.p. (°)
*Methyl (1:2-dihydro-2-acetoxy-1-naphthyl tri-*O*-acetylglucosid)uronate	Rabbit	+94	209
*Methyl (1-naphthyl tri-*O*-acetylglucosid)uronate	Rabbit	−79	160
*Methyl (2-naphthyl tri-*O*-acetylglucosid)uronate	Rabbit	−35	127–8
Methyl (1:2-dihydro-1-naphthyl tri-*O*-acetylglucosid)uronate	Rabbit	+23	203
	Rat	−84	203

* Corner *et al.* (1954).

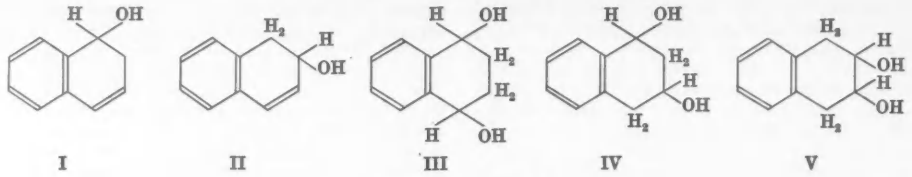

Fig. 1

naphthoresorcinol reaction. Three control experiments using solutions containing known amounts of naphthalene gave recoveries of 67, 72 and 76%.

Determination of structure

Possible formulae for acid-labile naphthalene precursors include formulae I–V (Fig. 1). 1:2-Dihydro-1-naphthol (I) has not been described, but 1:2-dihydro-2-naphthol (II) is dehydrated very rapidly to naphthalene in cold acid: naphthalene is not produced when 1:2:3:4-tetrahydronaphthalene-2:3-diol (V) is treated with boiling acid (Bamberger & Lodter, 1895).

Ultraviolet absorption spectra. The structure of the triacetyl methyl esters was further elucidated by a study of their ultraviolet absorption spectra. The rat and rabbit derivatives (in ethanol) had identical absorption spectra similar to that of 1:2-dihydronaphthalene (Fig. 2 and Table 2). The compounds possessing a 1:2:3:4-tetrahydronaphthalene structure (III–V) would be expected to have characteristic double peaked spectra with $\lambda_{max.}$ 267 and 275 mμ. and $\epsilon_{max.}$ (267 mμ.) 740. 1:4-Dihydronaphthalene has a very similar $\lambda_{max.}$ and $\epsilon_{max.}$ values to 1:2:3:4-tetrahydronaphthalene (Morton & de Gouveia, 1934), whereas 1:2-dihydronaphthalene has $\lambda_{max.}$ 262 mμ. and $\epsilon_{max.}$ 10 230. Beale & Roe (1951) observed that the optically active 1:2-dihydronaphthalene-1:2-diol had $\lambda_{max.}$ 265 mμ. and $\epsilon_{max.}$ 8250 (±2%). This indicates that the naphthalene precursors possess a 1:2-dihydronaphthalene structure.

Action of β-glucuronidase

When the crude dihydronaphthyl glucosiduronic acid fraction obtained from the partition column during the isolation was incubated at 37° with bacterial β-glucuronidase (Sigma Chemical Co., Missouri, U.S.A.), a steam-volatile oil was liberated which readily changed to naphthalene. To hinder this decomposition, solutions containing about 200 mg. of each crude naphthalene precursor were incubated at 37° with bacterial β-glucuronidase (1000 Sigma units/100 ml.) under benzene at pH 6·0 for 8 hr. The benzene did not inhibit the enzyme action but reduced bacterial contamination and stabilized the product. The benzene layer, after drying over anhydrous Na_2SO_4 and subsequent evaporation, yielded a trace of oil, which was steam distilled from alkaline solution. The steam distillate was extracted with ether, the ether was removed and the residue was steam distilled from acid when naphthalene was deposited in the condenser.

Identification of 1:2-dihydronaphthyl glucuronides

Bamberger & Lodter (1895) synthesized 1:2-dihydro-2-naphthol (m.p. about 35°) by treating 3-chloro-1:2:3:4-tetrahydro-2-naphthol with alkali; 1:2-dihydro-1-naphthol

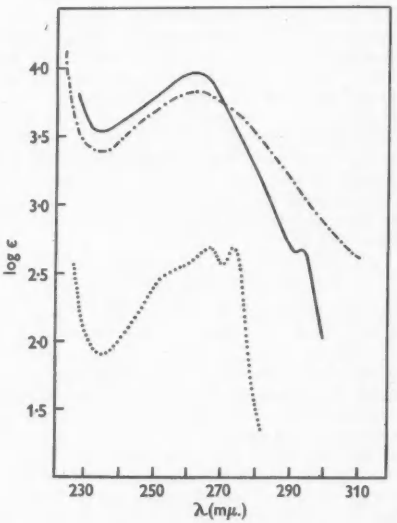

Fig. 2. The ultraviolet absorption spectra of: ——, 1:2-dihydronaphthalene; ·—·—·, methyl (1:2-dihydro-1-naphthyl tri-*O*-acetyl-glucosid)uronate; ····, 1:2:3:4-tetrahydronaphthalene.

Table 2. *Ultraviolet absorption data for some hydrogenated naphthalene derivatives*

Compound	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$
*1:2-Dihydronaphthalene	262	10 230
*1:4-Dihydronaphthalene	267	800
*1:2:3:4-Tetrahydronaphthalene	267	740
†1:2-Dihydronaphthalene-1:2-diol (synthetic racemate)	263	6 830
Crude barium salt of 1:2-dihydro-2-hydroxy-1-naphthyl glucosid-uronic acid (rat)	264	—
Methyl (1:2-dihydro-1-naphthyl tri-*O*-acetylglucosid)uronates (rat and rabbit)	263	6 770

* Morton & de Gouveia (1934).
† Beale & Roe (1951).

has not yet been synthesized. Alkaline permanganate oxidation of 1:2-dihydro-2-naphthol yielded products which were unsuitable for characterizing 1:2-dihydro-2-naphthol, of which no derivatives have been described. Attempted catalytic reduction of the 3:4 double bond of crude dihydronaphthyl glucosiduronic acid under various conditions to the corresponding tetralol derivative was unsuccessful; no decrease in absorption at 264 mμ. was observed.

Hydroxylation of the methylene group of 1:2-dihydro-1-naphthyl glucosiduronic acid would yield 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid which is readily converted into 1-naphthyl glucosiduronic acid in boiling acid (Corner *et al.* 1954). The dihydronaphthyl glucosiduronic acid was not oxidized with SeO_2 in boiling ethanol. Bromination in the 2-position of the crude glucosiduronic acid in water with *N*-bromosuccinimide (2%) and benzoyl peroxide (0·1%) for 3 hr. at room temp. followed by hydrolysis with 2% aqueous KOH for 4 hr. yielded sufficient 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid to be detected by paper chromatography. No naphthyl glucosiduronic acid could be detected after acid treatment of the solution.

Catalytic dehydrogenation. Wieland (1912) obtained naphthalene and tetralin by the disproportionation of 1:4-dihydronaphthalene at room temp. in the presence of palladium black. Similar experiments using a hydrogen donor as solvent have been made by Linstead, Braude, Mitchell, Woolridge & Jackman (1952), who obtained equal amounts of naphthalene and tetralin by heating 1:2-dihydronaphthalene at 65° with palladium–charcoal catalyst in tetrahydrofuran.

As a control experiment, 1:2-dihydronaphthalene (200 mg.) in ethanol (100 ml.) was shaken with 5% palladium on charcoal (500 mg.) at room temp. (the catalyst having been well washed with warm ethanol to remove impurities). The reaction was followed by plotting the ultraviolet absorption curves of suitably diluted samples of the reaction mixture at various time intervals. After 22 hr. about 75% of the dihydronaphthalene appeared to be converted into naphthalene. The palladium–charcoal catalyst was filtered off, and the solution evaporated to small volume and kept at 4°. After 2 days the solid was recrystallized 3 times from aqueous ethanol to yield naphthalene, m.p. 79°.

Under the same dehydrogenation conditions the dihydronaphthyl glucosiduronic acid fraction showed no appreciable reaction at room temp. after 6 days. When the experiment was repeated in boiling ethanol, a considerable drop in optical density at 264 mμ. occurred after 6 days, but no naphthyl glucosiduronic acid could be detected in the reaction mixture by paper chromatography. The lack of reactivity might have been due to impurities in the glucosiduronic acid fraction. However, when small quantities of pure methyl (1:2-dihydro-1-naphthyl tri-*O*-acetylglucosid)-uronate (rat metabolite) in ethyl acetate were treated in the same way, only a slight fall in optical density was observed. It was impossible in any of these experiments to obtain a characteristic curve of either the 1- or 2-naphthyl glucosiduronic acids (Fig. 3).

Dehydrogenation of dihydronaphthyl glucosiduronic acid fractions from rat and rabbit urines was eventually effected by using higher temperatures. The aqueous glucosiduronic acid fractions (20 ml. in each case) were adjusted to pH 8 and heated with palladium–charcoal catalyst (10 mg./ml.) in a sealed tube at 150° for 6 hr. The reaction mixtures, after filtering to remove the catalyst, showed a marked decrease in absorption at 264 mμ. Paper chromatograms of these solutions revealed that most of the dihydronaphthyl glucosiduronic acid had disappeared and that some 1-naphthol and 1-naphthyl glucosiduronic acid had been formed. The reaction mixtures were extracted with ether for 16 hr. to remove 1-naphthol and a portion of each solution was adjusted to pH 6·0 with acetate buffer and incubated at 37° for 5 hr. with bacterial β-glucuronidase (100 Sigma units/10 ml.). Samples of the ether-extracted enzyme hydrolysates were applied to Whatman no. 3MM paper and developed upwards for 2 hr. with 0·1 N-NH_4OH. This procedure, which completely separates 1- and 2-naphthol, showed the presence in each case of only 1-naphthol, which was detected by its characteristic fluorescence under ultraviolet light and colour reaction with diazotized *p*-nitraniline.

The *p*-toluidine salt of 1-naphthyl glucosiduronic acid (95 mg.) was isolated from the dehydrogenation reaction mixture of the rat metabolite using the method of Berenbom & Young (1951). (No *p*-toluidine salt could be obtained from the reaction mixture from the dehydrogenation of the rabbit metabolite.) When the *p*-toluidine salt was dissolved in 6 N-HCl (1 ml.) and the solution cooled to 4°, 1-naphthyl glucosiduronic acid separated. It crystallized from water in needles (10 mg.) which after drying at 100° *in vacuo* over P_2O_5 had m.p. 200° (decomp.), unchanged in admixture with authentic 1-naphthyl glucosiduronic acid (m.p. 198–200°) prepared by the method of Berenbom & Young (1951); $[\alpha]_D^{22} - 84{\cdot}3 \pm 2°$ in water (*c*, 1). (Found: C, 55·6; H, 5·8. Calc. for $C_{16}H_{16}O_7$, 1·5 H_2O: C, 55·3; H, 5·5%.) The ultraviolet absorption spectra (Fig. 3) corresponded to that of pure 1-naphthyl glucosiduronic acid ($\lambda_{max.}$ 282 and 319 mμ.; $\epsilon_{max.}$ 5650 and 1920). It thus appears that the two optically active dihydronaphthyl glucosiduronic acids are 1:2-dihydro-1-naphthyl glucosiduronic acids.

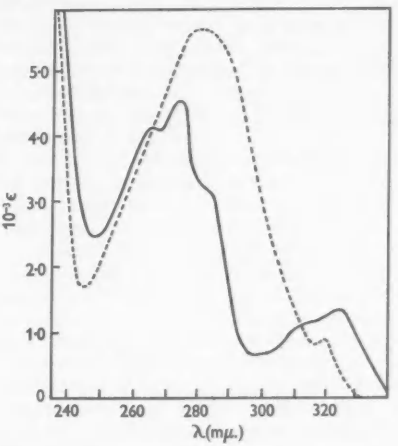

Fig. 3. The ultraviolet absorption spectra of: - - - -, 1-naphthyl glucosiduronic acid obtained by dehydrogenation of 1:2-dihydro-1-naphthyl glucosiduronic acid and ——, 2-naphthyl glucosiduronic acid obtained from the urine of rats dosed with 2-naphthol.

Possible precursors of 1:2-dihydro-1-naphthyl glucosiduronic acid

Alcohols administered to animals are often excreted as glucosiduronic acids. The chemical processes involved in the production of dihydronaphthol by animals dosed with naphthalene are as yet unknown, but free dihydronaphthol is probably first formed in the body from naphthalene and then conjugated with glucuronic acid. 1:2-Dihydro-1-naphthol might be formed in the body either by the addition of the elements of water to the 1:2-bond of naphthalene or by the monohydroxylation of 1:2-dihydronaphthalene.

1:2-Dihydronaphthalene was purified by the method of Rowe (1921) and was shown to be free of naphthalene by infrared analysis. A 50% (v/v) solution of 1:2-dihydronaphthalene in arachis oil (0·5 ml./rat) was administered to rats (200 g.) daily for 3 days. The rat urine was examined by paper chromatography and found to contain relatively more dihydronaphthyl glucosiduronic acid and much less free diol than did rat naphthalene urine. These urines yielded naphthalene and an oil when steam distilled from acid solution.

DISCUSSION

Many workers have shown that in the metabolism of naphthalene an acid-labile metabolite was formed which gave the parent hydrocarbon on subsequent acidification of the urine. The isolation of methyl (1:2-dihydro-1-naphthyl tri-*O*-acetylglucosid)uronate, which yields naphthalene on treatment with acid, has revealed a new class of compound formed during the metabolism of naphthalene (Boyland & Solomon, 1953). Neither 1:2-dihydro-1-naphthol nor any of its derivatives appear to have been previously reported.

1:2-Dihydronaphthalene-1:2-diol is a well established metabolite of naphthalene (Young, 1947; Booth & Boyland, 1949), yet nothing is known of the mechanism of its formation in the body. The occurrence of both mono- and di-hydroxy derivatives of 1:2-dihydronaphthalene in the urine of animals dosed with naphthalene suggested that an initial reduction of the 1:2-position in the naphthalene structure may be followed by hydroxylation at the reduced positions. This theory has been tested by injection of 1:2-dihydronaphthalene into rats when the glucosiduronic acid of 1:2-dihydro-1-naphthol and free 1:2-dihydronaphthalene-1:2-diol were detected in the urine. As free 1:2-dihydronaphthalene-1:2-diol was only present in small amounts in these urines, it seems probable that this metabolite is mainly formed from a precursor other than 1:2-dihydronaphthalene, and lends support to the 'perhydroxylation' theory. The dihydronaphthol and the diol are probably formed by separate metabolic routes. Although the 1:2-diol is excreted both in free and conjugated forms in the urines of rats dosed with naphthalene, 1:2-dihydro-1-naphthol is excreted only in the form of glucosiduronic acid.

SUMMARY

1. Acid-labile hydrocarbon precursors excreted in the urine of rats and rabbits dosed with naphthalene have been isolated as methyl (1:2-dihydro-1-naphthyl tri-*O*-acetylglucosid)uronates. The product from rat urine had $[\alpha]_D^{22} - 84°$ and that from rabbit urine + 23°.

2. The position of the hydroxyl group of these glucosiduronic acids was determined by catalytic dehydrogenation to 1-naphthyl glucosiduronic acid.

3. 1:2-Dihydronaphthalene also appeared to be excreted as a dihydronaphthyl glucosiduronic acid and so may be an intermediate in the metabolic production of this glucosiduronic acid from naphthalene.

We are indebted to Dr Peter Sims for the sample of 1:2-dihydronaphthalene, to Mr S. F. D. Orr for the infrared analysis, to Mr J. A. Marsh for technical assistance with injection of the rats, and to Mr D. Manson for the sample of 1-naphthyl glucosiduronic acid. This investigation has been supported by grants to the Royal Cancer Hospital and Chester Beatty Research Institute from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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Detection of Pyrrolidone Carboxylic Acid

By N. ELLFOLK* and R. L. M. SYNGE
The Rowett Research Institute, Bucksburn, Aberdeenshire

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In the course of work on low-molecular constituents of plant extracts which yield amino acids on hydrolysis (cf. Synge, 1951; Synge & Wood, 1954), the question has arisen of the extent of occurrence of pyrrolidone carboxylic acid (5-oxopyrrolidine-2-carboxylic acid, pyroglutamic acid) (PCA). We have encountered this substance and partially isolated it from among the acidic components of juice from grass which had been stored at $-20°$ for several months. We have since satisfied ourselves that PCA is at most a minor constituent of the 'bound amino acid' fraction of freshly prepared juice. In the course of this work we have observed some properties of PCA which are useful for detecting it at low concentration in biological material and which could serve as a basis for its determination. We are therefore reporting these observations independently of the progress of the work on other forms of 'bound amino acid'.

PCA, arising by decomposition of glutamine, is present in considerable amount in molasses and presumably also in cooked foods, silage, etc. Its failure to give a colour with ninhydrin and other specific chemical reactions is probably responsible for the sparseness of present knowledge of its natural distribution and metabolic behaviour.

The early isolations of PCA from biological material were by ether extraction (Staněk, 1912, 1913, 1915; Škola, 1920; Dolinek, 1927) or by extraction of precipitates or evaporation residues with glacial acetic acid (Foreman, 1914; Bethke & Steenbock, 1923; Greenberg & Schmidt, 1936). More recently, extraction of acidified solutions with ethyl acetate has usually been employed (Wilson & Cannan, 1937; Pucher & Vickery, 1940; Leuthardt, 1940; Woodward & Reinhart, 1942; Ratner, 1944; Kögl, Barendregt & Klein, 1948; Bray, James, Raffan & Thorpe, 1949). Pucher & Vickery (1940) proposed determination of glutamine as PCA formed after heating plant extracts at pH 6·5 at 100° for 2 hr. The PCA was then extracted into ethyl acetate and determined as amino N (Van Slyke nitrous acid procedure) after acid hydrolysis (see also Sorgato & Sorenzo, 1950). However, as Pucher & Vickery reported no blank determinations and used heated extracts of dried plant tissues, their results have little bearing on the natural occurrence of PCA. Moreover, our own experience is that conjugated forms of glutamic acid other than PCA, as well as other conjugated amino acids, are extracted by ethyl acetate from acidified extracts of fresh plant material. Neuberger & Sanger (1942) could not obtain satisfactory results using the method of Pucher & Vickery (1940) with potato extracts.

The procedure of Schlütz (1953), which involves hydrolysis of constituents of urine soluble in aqueous ethanol, is equally open to criticism for lack of specificity.

More recently chromatography has been used for detecting or determining PCA. Kögl *et al.* (1948) mention chromatography in ethanol on alumina. Stark, Goodban & Owens (1950, 1951) and Owens, Goodban & Stark (1953) have used chromatography on ion-exchange resins. Stark *et al.* (1950; cf. Goodban, Stark & Owens, 1953), Le Quesne & Young (1952), Rowlands & Young (1952) and Simonart & Chow (1953) have used partition chromatography on paper, spraying with an indicator to locate the PCA. See also Pichler (1952).

Massa (1935) proposed a polarimetric method for determining PCA in molasses. Kanao (1948) has used a reaction of PCA with formaldehyde and formic acid for its determination. Hasse & Schumacher (1950) observed enzymic decarboxylation of PCA by preparations from radish, but gave no experimental details.

We have observed: (*a*) that PCA can conveniently be detected on filter paper by the chlorine–starch–iodide reaction of Rydon & Smith (1952); (*b*) that PCA has an anionic mobility at pH 6–7 exceeding those of glutamic acid or of any of the other components of grass juice which give a positive chlorine–starch–iodide reaction; (*c*) that PCA, as the free acid, is readily extractable into the phenol-rich phase of the system phenol–water and gives a zone occupying a characteristic position on water–phenol partition chromatograms (phenol phase stationary). We have further confirmed the observations of Le Quesne & Young (1952) and Rowlands & Young (1952) on filter-paper chromatography of PCA with *n*-butanol–water.

In view of our observations on the water–phenol distribution of PCA it seems likely that the observations of Austerweil & Delort-Stachenko (1952)

* Present address: Biochemical Institute, Helsinki, Finland.

relate not, as they supposed, to PCA but to some other substance. PCA would be expected to move faster than glutamic acid on phenol–water filter-paper chromatograms; nor can it be revealed with ninhydrin.

In order to ascertain if PCA occurs in fresh grass, we took freshly expressed juice, acidified to pH 2, and extracted it with phenol in a simple countercurrent train. Such a procedure yields in the phenol extract most of the 'bound amino acid' constituents (cf. Synge & Wood, 1954). Filter-paper electrophoresis showed that no more PCA was present in the phenol extract than might be expected from decomposition of the glutamine present under the acid conditions of the extraction (cf. Thierfelder & von Cramm, 1919; Melville, 1934; Ratner, 1944). PCA added initially to the juice was recovered in apparently good yield. PCA, derived from glutamine, was likewise found on applying the same procedure to juice which had been heated at neutral pH.

In view of the amount of PCA in freshly prepared grass juice being at most small compared with other forms of 'bound amino acid', we have not worked out any procedure for its exact determination. However, some zone-electrophoretic technique (see Tiselius & Flodin, 1953) could be adapted to take advantage of PCA being the nitrogenous compound which migrates most rapidly towards the anode at neutral pH. This differentiates PCA from other forms of 'bound glutamic acid' extractable with organic solvents, such as those at present being studied as well as, presumably, such known compounds as pteroylglutamic acid, phenacetylglutamine, etc. Extraction from acidic aqueous solution into organic solvent is clearly desirable for preliminary concentration of PCA and elimination of free glutamic acid, plant acids, carbohydrates, etc. However, this must not be done in the presence of glutamine, in view of its decomposition to PCA under such conditions. Glutamine would probably be best eliminated by initially isolating acidic constituents from the crude extract on anion-exchangers or by electrical transport in a diaphragm cell at neutral pH.

EXPERIMENTAL

L-*Pyrrolidone carboxylic acid* (*PCA*)

Partially racemized PCA was prepared by heating 15 g. L-glutamic acid ($[\alpha]_D^{25}+30{\cdot}3°$ in 9% (w/v) HCl, $c=2$, $l=0{\cdot}5$) for 2 hr. at 150–158° (cf. Abderhalden & Kautzsch, 1910). The melt was crystallized from 15 ml. water. Yield, 3·9 g. For analysis, the product was dried *in vacuo* at room temp. over H_2SO_4 and soda lime. $[\alpha]_D^{25{\cdot}5}-5{\cdot}4°$ in water ($c=1{\cdot}96$, $l=0{\cdot}5$). N (Kjeldahl), 10·5. Calc. for $C_5H_7O_3N$: N, 10·9%. Carboxyl N (conditions of Synge, 1951), < 1% of total N. No colour was observed after the heating with ninhydrin. When a hydrolysate (24 hr. in 6N-HCl at 105°) was evaporated to dryness and analysed under the same conditions, carboxyl N was 110% of total N. Presumably the low optical rotation was due to not collecting a high yield of crystals, to avoid contamination with glutamic acid. Abderhalden & Kautzsch (1910) noted that the DL compound is less soluble than the L enantiomorph.

Detection and partial isolation of PCA in extract of stored grass

Countercurrent distribution of diffusate with phenol–water. Italian rye grass which had been stored for 4 months at −20° (11 kg.) yielded on treatment with ether as before 1560 ml. of juice; this was dialysed in cellophan against chloroform water (10 l.) for 5 days at 0–2° (cf. Synge, 1951). The diffusate (9·6 l. containing 1·45 g. N) was evaporated *in vacuo* to 200 ml. This was placed in the first of a train of five separating funnels; the other four contained each 200 ml. of the water-rich phase of the system water–phenol (freshly distilled from A.R. grade). Countercurrent distribution was effected by passing eight 200 ml. portions of the phenol-rich phase of the same system through the train. Separation of the phases was hastened by centrifuging when necessary. The eight portions of phenol-rich phase emerging from the train were pooled and evaporated *in vacuo* with repeated addition of water (cf. Synge & Wood, 1954).

Fractionation in diaphragm cell. The residue was taken up with water, adjusted to pH 6–7 with NH_3 and fractionated (two batches) in the four-compartment diaphragm cell according to Synge (1951). Material from the acetic acid compartment contained 0·83% of the N of the original diffusate. This was evaporated to dryness *in vacuo* repeatedly with addition of water to remove acetic acid (fraction P4A).

Water-phenol partition chromatography. A portion of P4A (10·55 mg. N, 605 mg. dry matter) was taken up in 8·5 ml. of water-rich phase of the above phenol–water system and chromatographed (cf. Synge & Wood, 1954) on a column (5·4 cm. diam., 10·8 cm. long) made from 87 g. kieselguhr (Hyflo Supercel, Johns Manville Co., London), which had been treated with dichlorodimethylsilane according to Howard & Martin (1950), mixed with 58 ml. phenol-rich phase and packed as a slurry in water-rich phase using the perforated-disk tool of Howard & Martin (1950). The chromatogram was developed with water-rich phase in a cool room (14°) without special temp. control. Successive fractions of effluent were collected, weighed and evaporated in tared flasks to dryness *in vacuo* below 40°, with repeated addition of water until phenol was removed. The residues were weighed and the distribution of dry matter in the effluent was plotted. Since the resulting curve showed no well-marked peaks, the fractions were pooled into six successive groups accounting for roughly equal portions of the dry matter (and N) recovered. Of these, group 3 (P4A-3), comprising the effluent from the column (counted from moment of applying sample for analysis) between 253 g. and 373 g. of effluent, contained 106·4 mg. dry matter and 3·08 mg. N.

Filter-paper ionophoresis. Material from the various chromatographic fraction-groups was subjected to filter-paper ionophoresis in 0·04M sodium phosphate buffer (pH 7·1) for 4 hr. at 10 v/cm. according to Kunkel & Tiselius (1951), using glass plates smeared lightly with Silicone Fluid M.S. 550 (Hopkin and Williams Ltd., Chadwell Heath, Essex) and sealed at the edges with Dow Corning Stopcock Grease (Dow Corning Corpn., Midland, Mich.). Three sheets of paper were used in each run, the same samples (*ca.* 1 mg.

dry matter in 5 μl. water) being applied at corresponding positions on each sheet. It was found that the silicone interfered with the Cl_2–starch–KI reaction of Rydon & Smith (1952). Accordingly, the middle sheet was always used for this test.

Material from each of the fraction-groups 1–6 on ionophoresis showed anionic material of varying mobilities giving positive Cl_2–starch–KI reaction. However, only P4A-3 showed the presence of a strong spot migrating faster towards the anode than any of the other Cl_2–starch–KI reacting components of any of the six fraction-groups. This component gave no colour with ninhydrin. Its isolation was attempted as follows.

Silica-jelly ionophoresis. Procedure *B* of Consden, Gordon & Martin (1946) was adopted, using a trough 55 cm. long, 4 cm. broad and 0·7 cm. deep. For the main jelly, 9·4 ml. of a 1:2 (by vol.) dilution of waterglass (140° Tw., Jos. Crosfield Ltd., Warrington) was diluted with water and neutralized (bromothymol blue) with acetic acid to final vol. 150 ml. The inlay (near cathode end of jelly) was made by neutralizing a sample from P4A-3 (80 mg. dry matter, 2·32 mg. N) with diluted waterglass to give the same pH and silica concentration. The cathode was perfused with a solution of 0·22 M sodium acetate in 0·011 M acetic acid and the anode with 0·22 M sodium acetate. 100 mA was allowed to pass through the apparatus (without special cooling precautions) for 10·75 hr. A print was then taken and coloured with Cl_2–starch–KI. This revealed the centre of the main zone 15·5 cm. to the anode side of the inlay. Two other, obviously complex, zones occupied most of the space between the inlay and the principal zone. The jelly from 12·5–19·0 cm. on the anode side of the inlay was cut out. Obvious impurities present in this section (not coinciding exactly with the principal zone) were a trace of brown pigment, a zone fluorescing pale blue in u.v. light and a zone giving a purple colour on treating the print with Cl_2 (before spraying with starch–KI).

The cut-out slab of jelly was stirred with water and the resulting slurry transferred to the specimen compartment of the four-compartment diaphragm cell. The fractionation was as usual (pH 6–7) except that NaOH was used instead of NH_3 in the cathode compartment and for maintaining pH in the specimen compartment. After 6 hr. the contents of the acetic acid compartment was removed and evaporated to dryness *in vacuo* repeatedly with water. The residue was taken up in water, filtered from a little insoluble material (silica) and again evaporated (11·0 mg. dry matter; 0·54 mg. N, representing 6·8% of the N of P4A, i.e. 0·057% of the N of the original diffusate). Subsequent work showed that the very low yield of PCA was due to not having acidified the diffusate before the phenol extraction.

Characterization of partially isolated PCA

The product, analysed after drying *in vacuo* over H_2SO_4–soda lime at room temp., consisted of crystals embedded in syrup and was freely soluble in water. $[\alpha]_D^{19} - 11{\cdot}3°$ in water ($c = 1{\cdot}7$, $l = 0{\cdot}5$); N (Kjeldahl), 4·95%; acid equiv. wt. 237. Carboxyl N was $< 1\%$, but after hydrolysis 87%, of total N (conditions as above; no colour on heating unhydrolysed material with ninhydrin). The acid hydrolysate on 2-dimensional filter-paper chromatography (conditions of Synge, 1951) showed a strong spot corresponding to glutamic acid and very faint spots corresponding to glycine and other neutral amino acids.

Filter-paper ionophoresis (as above) showed with Cl_2–starch–KI a main spot identical in mobility (mixed spot) with authentic PCA. The product from grass also showed a faint spot migrating 0·83 times as fast as the main spot. PCA had mobility towards the anode, measured from a spot of *N*-2:4-dinitrophenylethanolamine which served as a control for electroendosmosis, etc. (Mould & Synge, 1954), 1·13 times that of L-glutamic acid. The authentic PCA preparation gave a faint spot, having 1·24 times the anionic mobility of the main spot, which may be due to a dimeric anhydride of glutamic acid.

On descending chromatography with *n*-butanol–water on Munktell OB paper (Grycksbo Pappersbruk AB, Grycksbo, Sweden), the product from grass and PCA behaved identically (confirmed by mixed chromatogram). The spots (Cl_2–starch–KI) had leading edge at R_F *ca.* 0·5 and diffuse tails (cf. Le Quesne & Young, 1952; Rowlands & Young, 1952).

Authentic PCA was chromatographed on a water–phenol column as above (1 cm. diam.; 3 g. silane-treated kieselguhr). The Cl_2–starch–KI reaction was applied to spots on filter paper from successive effluent fractions. The peak of the zone was found at $R = 0{\cdot}73$, the leading edge of the zone being much more diffuse than the tailing edge. The corresponding chromatographic fraction in the isolation (P4A-3) represented the cut $R = 0{\cdot}98$ to $R = 0{\cdot}66$. $R = 0{\cdot}73$ was calculated (Martin & Synge, 1941) to correspond to partition coefficient $\alpha = 3{\cdot}0$ in favour of the stationary phenol-rich phase of the system phenol–water.

We conclude that the isolated product was PCA contaminated with much N-free but only little nitrogenous impurity.

Detection of PCA in fresh grass juice

Control experiments in which PCA was added to grass juice showed that recovery in the phenol extracts was inadequate unless the juice was acidified before extraction.

Juice was squeezed from freshly picked Italian rye grass after treatment with ether, ether was removed by a short evaporation *in vacuo* in the cold and the juice was acidified to pH 2 (glass electrode) with 6 N-H_2SO_4. Two 100 ml. portions of acidified juice (each 124 mg. total N) were taken. To one was added PCA (1·07 mg. N). The portions were then separately subjected to the countercurrent distribution as described above, using 100 ml. portions of phenol–water phases. The extraction lasted approx. 4 hr. It was done at room temp. since the crystallization of a phenol hydrate prevented working at 0–2°.

A suitable portion of the combined phenol extract from each distribution was evaporated as above and the residues were taken up with water to give a slurry which was applied for filter-paper ionophoresis of spots. Each spot represented 153 μg. N of original grass juice, and, for the specimen with added PCA, 1·32 μg. N of added PCA. A control spot (1·32 μg. N of PCA) was run in parallel. After ionophoresis as described above, Cl_2–starch–KI treatment revealed a streak of staining material which had migrated towards the anode, as well as much neutral staining material in the grass extracts. None of this material migrated faster than PCA; the spot in the corresponding region, though present, was much less intense than the PCA control spot with the extract of untreated juice, whereas the extract of juice to which PCA had been added showed a spot in this region of comparable intensity to the control spot. The PCA present in

the fresh juice was therefore much less than 0·86% of the N of the juice. Assuming 1–2% decomposition of the glutamine present (cf. Thierfelder & von Cramm, 1919; Melville, 1934; Ratner, 1944) and using the figure for glutamine given below, 0·1–0·2% of the total N of the juice might be expected to have been transformed into PCA.

The juice was also adjusted to pH 6·8 with NaOH and heated at 100° for 3 hr. It was then subjected to acidification, phenol extraction and filter-paper ionophoresis as above. A spot much more intense than the PCA control spot was found in the same position. Glutamine was determined by estimating carboxyl N on the heated and unheated juice (procedure of Synge, 1951. Cf. Neuberger & Sanger, 1942; Hamilton, 1945). Carboxyl N was 41·8% of total N originally, 30·5% after heating. This corresponds to glutamine N 22·6% of total N (cf. Synge, 1951), and to the formation of PCA amounting to 11·3% of the total N of the grass juice.

SUMMARY

1. Observations on ionophoresis and partition chromatography of pyrrolidone carboxylic acid (PCA) are reported. PCA is conveniently detected by the procedure of Rydon & Smith (1952). PCA was detected in, and partially isolated from, Italian rye grass that had been stored at −20°.

2. In extracts of fresh grass the amounts of PCA are negligible compared with those of other forms of 'bound amino acids'.

3. Methods hitherto used for detecting and determining PCA are reviewed and possible improvements are suggested.

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The Urinary Excretion of Vitamin A by the Dog

By A. N. WORDEN, J. BUNYAN and A. W. DAVIES
Cromwell House, Huntingdon

and C. E. WATERHOUSE
Research Laboratories, Bob Martin Ltd., Southport

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Catel (1938) reported that vitamin A was present in appreciable amounts in the urine of normal dogs, although absent from that of normal human beings, rabbits and rats. In four dogs (two male and two female, varying in body weight from 7 to 31·5 kg.) from which he obtained seven spontaneous or catheterized samples, the concentration of vitamin A ranged from 0·2 to 2·0 mg. (66 to 660 i.u.) per 100 ml. In two samples a proteinuria was detected but no other abnormalities were recorded. Lawrie, Moore & Rajagopal (1941) found that random samples of urine obtained from an apparently healthy male Fox Terrier contained from 90 to 450 i.u. per 100 ml., the latter value being equal to the highest recorded in human patients with pneumonia.

EXPERIMENTAL

In the course of our experiments on the urinary excretion of B vitamins by the dog (Worden, Waterhouse & Partington, 1952, 1954; Worden & Waterhouse, 1955) we have taken the opportunity of determining the vitamin A content of 24 hr. urine samples voided by four dogs in metabolism cages. The animals were all healthy specimens that had been maintained under known dietary conditions for periods of from 1 to 3 years, and were adapted to the experimental procedures involved. The urine samples were extracted by the method described by Lawrie *et al.* (1941), and the vitamin A was estimated by the method of Carr & Price (1926) with the aid of a Unicam diffraction-grating spectrophotometer calibrated against vitamin A acetate. In some instances a pink fluorescence developed on evaporation of the ether extract. The responsible substance was, however, found to be water

Table 1. *Vitamin A content of dog urine*

Experimental animal	Urine sample collected 24 hr. ending	Vitamin A content of urine: i.u./100 ml.	Vitamin A content of urine: Total excretion i.u./24 hr.
'Pat': 4 yr. old male smooth-coated mongrel Terrier; weight 22·2–22·6 kg.	6. v. 53	6·5	37·0
	7. v. 53	4·5	27·2
	23. iii. 54	7·5	43·5
	26. iii. 54	7·5	72·7
	25. v. 54	3·75	23·2
'Scottie': 3 yr. old male Scotch Terrier; weight 8·6–9·5 kg.	19. iv. 53	6·9	45·1
	22. iv. 53	2·5	14·3
	24. iv. 53	6·0	25·2
	16. ii. 54	13·3	56·0
	14. v. 54	15·2	101·0
	21. v. 54	24·0	134·0
'Roxy': 6 yr. old female black-and-white Cocker Spaniel; weight 12·2–14·5 kg.	20. i. 54	21·2	89·5
	21. i. 54	16·2	108·0
	22. i. 54	14·3	95·0
	26. i. 54	7·1	44·0
	29. i. 54	4·8	40·0
	5. ii. 54	4·2	10·9
	12. ii. 54	2·7	18·6
	23. iv. 54	17·5	89·5
	28. iv. 54	47·5	235·0
	30. iv. 54	25·8	180·0
	3. viii. 54	12·5	46·2
	4. viii. 54	4·2	28·4
'Dusty': 2 yr. old male Husky; weight 32·7 kg.	23. viii. 54	12·5	73·4
	2. ix. 54	3·7	36·0
	8. ix. 54	6·75	58·7
	9. ix. 54	1·75	17·0

soluble and to be removed completely by a further saponification followed by re-extraction with ether. The results are given in Table 1.

DISCUSSION

Lawrie *et al.* (1941) believed that the fluctuations in the urinary vitamin A level of the dog studied by them were determined by factors other than the supply immediately available from the diet. This seemed to be true for the much smaller fluctuations noted by us, for in all the observations other than those involving the two samples collected on 5 and 12 February 1954 from 'Roxy' the animals were receiving standard amounts of a cooked rabbit meat–wholemeal bread–milk diet as described elsewhere (Worden *et al.* 1952) and containing from 15 to 21 i.u. vitamin A per ounce. Prior to the experiment in which the first five values for 'Roxy' were recorded, this animal had received considerable quantities of a special diet containing carotene equivalent to at least 2000 i.u. vitamin A per ounce, and it was thought that this might have accounted for the initial somewhat higher levels of vitamin A excreted in her urine. For the experiment during which the two observations on 5 and 12 February 1954 on this animal were made, however, the same carotene-rich diet was fed throughout, but there was no apparent effect on the level of urinary excretion of vitamin A.

These results confirmed that small quantities of vitamin A were present in the urine of normal dogs, but the levels obtained were of a much lower order than those recorded by Catel (1938) and by Lawrie *et al.* (1941). It is known that there are marked variations in the liver vitamin A reserves of apparently healthy dogs (Bradfield & Smith, 1938; Linton & Brownlee, 1939), but the excretion of very large amounts in the urine is suggestive of some pathological condition. There is a high incidence of nephritis among domestic dogs, and although clinical recovery may occur, with survival for many years, tests of renal efficiency in such animals, or measurements of arterial pressure (McCubbin & Corcoran, 1953) will indicate some impairment of function. Alternatively, the urinary excretion of vitamin A may be linked with the high plasma levels of the vitamin attained in this species (Beher & Gaebler, 1950).

In samples (24 hr.) of urine obtained from a cat confined to a metabolism cage, no detectable amount of vitamin A was found.

SUMMARY

Vitamin A levels have been determined in 24 hr. urine samples collected from four normal dogs in metabolism cages. The levels have been found to range from 2·5 to 47·5 i.u. vitamin A per 100 ml., or 14·3–235·0 i.u. per day.

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